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(54) Title: REGULATABLE ELIMINATION OF GENE EXPRESSION, GENE PRODUCT FUNCTION AND ENGINEERED HOST CELLS			
(57) Abstract  Materials and methods are disclosed for regulated obstruction of the expression of a target gene or the biological effect of its gene product in genetically engineered cells or organisms containing them. Aspects of the invention are exemplified by recombinant modifications of host cells and their use <i>in vitro</i> and <i>in vivo</i> for the regulatable blockade of expression of a target gene, for interference with the function or effect of a target gene product or for the regulatable elimination of a target gene.			

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## Regulatable Elimination of Gene Expression, Gene Product Function and Engineered Host Cells

### Technical Field

5           This invention concerns materials, methods and applications relating to the multimerizing of chimeric proteins with a dimeric or multimeric, preferably non-peptidic, organic compound. Aspects of the invention are exemplified by recombinant modifications of host cells and their use *in vitro* and *in vivo* for the regulatable blockade of expression of a target gene, for  
10 interference with the function or effect of a target gene product or for the regulatable elimination of a target gene. The materials, methods and applications of the invention provide a means for regulating expression of genes introduced by recombinant modifications of host cells, including completely eliminating a population of genetically engineered cells, thus  
15 providing a fail-safe mechanism for controlling genetically engineered cells used in gene therapy.

### Background

One approach to studying the role of a gene in a host organism is to eliminate it or replace it with a dysfunctional counterpart. This can be  
20 accomplished, for example, by genetic engineering of cultured embryonic stem ("ES") cells followed by introduction of the engineered cells into embryos which develop into whole organisms lacking a functional copy of the gene. Typically such experiments are conducted in mice. However, where the gene product is required for normal development of the host organism, simply  
25 eliminating, or "knocking out", the gene may yield severely dysfunctional animals, which may not be useful, or no animals at all.

A number of systems have been developed for creating "conditional knock-outs", i.e., cells or organisms in which a gene can be ablated when desired, i.e., at or after any desired stage of development and for creating cell-

type specific (non-conditional) knock-outs, i.e., organisms in which a gene is ablated in a specific type of cell. See for example, Watson et al., *Recombinant DNA*, 2d ed. (1992), especially Chapters 14 and 24; Gu et al., *Science* 265 (1994) 103-106; Barinaga, "Research News: Knockout Mice: Round Two," *Science* 265 (1994) 26-28; Lasko et al., "Targeted oncogene activation by site-specific recombination in transgenic mice," *Proc. Natl. Acad. Sci. USA* 89 (1992) 6232-36; and Orban et al., "Tissue- and site-specific DNA recombination in transgenic mice," *Proc. Natl. Acad. Sci. USA* 89 (1992) 6861-65.

Independent of that work, biological switches have been developed which are based on ligand-mediated multimerization of immunophilin- and other receptor-based recombinant proteins. Aspects of that work are disclosed in Spencer et al., *Science* 262 (1993) 1019-1024 and International Patent Applications PCT/US94/01617 and PCT/US94/08008, the contents of all three of which are incorporated herein by reference.

Intracellular crosslinking of chimeric proteins by synthetic ligands has potential in basic investigation of a variety of cellular processes, in regulating the synthesis of proteins of therapeutic or agricultural importance, including the regulatable obstruction of the expression or function of these genes and in regulatably initiating cell death in engineered cells. Furthermore, ligand mediated oligomerization now permits regulated gene therapy. In so doing, it provides a fresh approach to increasing the safety, expression level and overall efficacy obtained with gene therapy.

Illustrative publications disclosing further background information of interest are provided in PCT/US94/01617, especially on pages 1-4. However, as will be clear from this disclosure, none of the foregoing publications describe or suggest the present invention.

### Summary Of The Invention

This invention provides materials and methods for the genetic engineering of host cells to render the cells, their progeny and organisms containing them, susceptible to blockade of expression of a selected gene, to interference with the functioning of the gene product or to elimination or

inactivation of a selected gene, in a regulated fashion. The invention further provides materials and methods for genetic engineering of host cells to render the cells and their progeny susceptible to regulated, programmed cell death, otherwise known as apoptosis. Such cells, as well as organisms containing them, are useful as biological reagents for a variety of research purposes, as described *infra*, including the study of diseases characterized by the dysfunction or absence of a gene or gene product of interest. Table 1 below provides a non-exclusive, illustrative list of diseases linked to the dysfunction or absence of a gene or gene product.

The invention involves the adaptation of a method and materials for using homo- and hetero-multimerization of chimeric "responder" proteins to trigger gene transcription in living cells. As used herein, the terms *multimer*, *multimerize* and *multimerization* encompass dimers, trimers and higher order multimers and their formation. The chimeric responder proteins are intracellularly expressed fusion proteins which contain a specific receptor domain and are responsive to the presence of a corresponding multimerizing agent. The multimerizing agent is a multivalent ligand which is capable of binding to more than one of the chimeric protein molecules to yield dimers or higher order multimers of the chimeras. The chimeric proteins are designed such that the ligand-mediated multimerization triggers transcription of a gene under the transcriptional control of an element which is responsive to such multimerization. This gene may be a "blocking" gene, which encodes a blocking factor such as an anti-sense message complementary to, and capable of interfering with transcription of, a target gene or a ribozyme, which is capable of preventing the expression of a target gene. Alternatively, the blocking gene may encode an antibody moiety, such as a single-chain antibody, which is capable of binding to and preferably neutralizing or blocking a biological effect of the gene product of the target gene.

Table 1

	Disease	Gene/Gene Product
	Parkinson's disease	tyrosine hydroxylase
	neural degenerative diseases	growth factors or myelinating proteins
5	osteoporosis	bone morphogenic factors
	anemia	erythropoietin
	thrombocytopenia	thrombopoietin
	immunodeficiencies	antigen receptors, immune cell signaling proteins
	cancer	tumor suppressor genes in animals carrying suppressor mutations
10	cystic fibrosis	CFTR
	diabetes mellitus	insulin, insulin receptor
	pituitary dwarfism	growth hormone
	emphysema	alpha1-antitrypsin
	familial hypercholesteremia	LDL receptor
15	thalassemia major and sickle cell anemia	beta-globin
	hemophilia A	Factor VIII
	hemophilia B	Factor IX
	Gaucher's disease	glucocerebrosidase
20	glycogen storage diseases	several types/genes
	phenylketonuria	phenylalanine hydroxylase
	severe combined immunodeficiency disease	ADA, purine nucleoside phosphorylase, p70 <sup>ZAP</sup>
25	Duchenne muscular dystrophy	dystrophin
	Lesch-Nyhan syndrome	Hypoxanthine phosphoribosyl transferase
	Lou Gehrig's Disease	superoxide dismutase
	Glanzmann thrombocytopenia	GP IIb-IIIa - the fibrinogen receptor
30	Tay-Sachs disease	hexosaminidase

The blocking gene also may encode a dominant negative gene product capable of blocking the effect of a target gene product, or it may encode a protein whose action will lead to the elimination of the selected target gene entirely. An example of a blocking gene whose action will lead to the elimination of a target gene is the gene encoding the protein Cre, which produces Cre recombinase. Expression of the Cre recombinase leads to elimination of a target gene appropriately flanked by "loxP" sequences in the host cells. Alternatively, the gene may be a gene which provides a means for eliminating a population of engineered cells, either growing in culture or *in vivo*, providing a mechanism for regulating and controlling engineered cells.

Thus, this invention involves one or more chimeric responder proteins, DNA constructs ("responder" constructs) encoding them, and multi-valent ligand molecules capable of multimerizing the chimeric proteins. The chimeric proteins contain at least one ligand-binding (or "receptor") domain and an action domain capable, upon multimerization of the chimeric protein molecules, of initiating transcription of the blocking gene or the elimination effector gene within a cell. The chimeric proteins may further contain additional domains. These chimeric responder proteins and the responder constructs which encode them are recombinant in the sense that their various components are derived from different sources, and as such, are not found together in nature (i.e., are mutually heterologous). Also provided are recombinant "blocking" constructs containing a blocking gene under the transcriptional regulation of a control element responsive to the presence of multimerized responder proteins described above. The transcriptional control element is responsive in the sense that transcription of the blocking gene is activated by the presence of the multimerized responder chimeras in cells containing these constructs. Exposure of the cells to the multimerizing ligand results in expression of the blocking gene. The constructs of this invention may contain one or more selectable markers such as a neomycin resistance gene (neo<sup>r</sup>) and herpes simplex virus-thymidine kinase (HSV-tk). When cells which have been genetically engineered to contain and express the responder and the blocking gene are exposed to the multimerizing ligand, expression of

the blocking gene is activated and expression of the target gene or functioning of the target gene product is impaired.

Recombinant "Cre" constructs encoding Cre under the transcriptional regulation of a control element responsive to the presence of multimerized responder proteins described above are also provided. The transcriptional control element is responsive in that transcription of Cre is activated by the presence of the multimerized responder chimeras in cells containing these constructs. That is, exposure of the cells to the multimerizing ligand results in expression of Cre. Cells may also contain a "target" gene, preferably, but not necessarily, an endogenous gene to be eliminated, which is flanked by loxP sequences ("floxed"). The floxed target gene is introduced into the cell, for example, by homologous recombination using a recombinant "target" construct containing part or all of a copy of the endogenous target gene and/or endogenous flanking DNA sequence thereof, together with loxP DNA elements. As in the case of other constructs of this invention, the floxed target gene construct may contain one or more selectable markers such as the neomycin resistance gene (neo<sup>r</sup>) or herpes simplex virus-thymidine kinase gene (HSV-tk). When cells, which have been genetically engineered to contain and express the responder, Cre and floxed target gene constructs, are exposed to the multimerizing ligand, expression of Cre is activated and the target gene is eliminated.

Additionally recombinant constructs encoding primary chimeric proteins of this invention permit ligand-regulated apoptosis. These chimeric constructs contain at least the cytoplasmic domain of the fas antigen or Apo-1 antigen, which when cross-linked, induces apoptosis in most cell types. (See Trauth et al. *Science* 245 (1989) 301-305; Watanaba-Fukunaga et al. *Nature* 356 (1992) 314). In this way one can provide for ligand-inducible cell death for an engineered population of cells.

Modified cells are produced by introducing the desired construct(s) into selected host cells. This may be accomplished using conventional vectors and techniques, many of which are commercially available. If desired, the modified cells into which one or more constructs have been successfully



introduced may then be selected, separated from other cells and cultured, again by conventional methods.

Where the target gene is an endogenous gene, incorporation of a target gene construct into a host cell may be effected by homologous recombination, by known methods such as that of Gu et al., 1994, Science 265:103-106. Alternatively, if endogenous copies of the target gene are deleted or rendered nonfunctional, by homologous recombination or mutation, the recombinant target gene construct may be introduced by any desired means. Incorporation of the responder constructs and Cre constructs may also be effected using conventional transfection vectors and techniques.

Genetically engineered cells, which can be grown together with other cells and which can be selectively regulated in, or ablated from, the mixture of cells by addition of an effective amount of an oligomerization ligand which is capable of binding to the primary chimeric protein, are an important aspect of this invention. Contacting the engineered cells with the oligomerization ligand can trigger the regulatable obstruction of the expression of a specific gene through the expression of a target gene which can block or interfere with the function of a target gene. Alternatively, the regulated gene can be one whose expression leads to specific elimination of a target gene, or cell death of the engineered cells. For example, such cells may be permitted to produce an endogenous or heterologous product for some desired period, and may then be deleted by addition of the ligand. In such cases, the cells are engineered to produce a primary chimera in accordance with this invention.

The cells, which may be further engineered to express a desired gene under ligand-induced regulation, may be grown in culture by conventional means. In that case, addition to the culture medium of the ligand for the optional chimera leads to expression of the desired gene and production of the desired protein. Expression of the gene and production of the protein can then be turned off by adding to the medium an oligomerization antagonist reagent, as is described in detail below. In other cases, production of the protein is constitutive. In any event, the engineered cells can then be eliminated from the cell culture after they have served their intended purpose

(e.g. production of a desired protein or other product) by adding to the medium an effective amount of the appropriate oligomerizing ligand to cause oligomerization of the primary chimera and induce apoptosis in the engineered cells. Engineered cells of this invention can also be used in vivo, to modify whole organisms, preferably animals, including humans, such that the cells produce a desired protein or other result within the animal containing such cells. Such uses include gene therapy. Alternatively, the chimeric proteins and oligomerizing molecules can be used extracellularly to bring together proteins which act in concert to initiate a physiological action.

To create transgenic animals containing modified cells of this invention the desired constructs are transfected into appropriate cell lines, for example, ES cells. Alternatively, the desired constructs may be directly microinjected into early embryos. See for example, Watson et al., Recombinant DNA, 2d ed., 1992, especially Chapters 14 and 24. In the latter case, use of a tissue-specific expression control sequence, such as a promoter or enhancer sequence, in the responder construct permits tissue-specific expression of the chimeric responder protein(s), which in turn permits tissue-specific, regulatable expression of the blocking gene, and thus tissue-specific blockade of the target gene or its gene product. It should be further noted that animals may be so produced which comprise cells containing and capable of expressing the responder. Other animals may be produced which comprise cells containing a blocking gene construct. Breeding the two types of engineered animals yields offspring which contain cells containing both of the foregoing constructs.

A tissue-specific expression control sequence (promoter/enhancer) in the responder construct also permits tissue-specific expression of the chimeric responder protein(s), which in turn permits tissue-specific, regulatable expression of Cre, and thus tissue-specific elimination of the target gene. It should be further noted that animals may be so produced which comprise cells containing and capable of expressing the responder and Cre constructs. Other animals may be produced which comprise cells containing a desired target gene construct. Breeding the two types of engineered animals yields offspring which contain cells containing all of the foregoing constructs. Animals and

their progeny may be conveniently characterized by conventional genetic analysis. In addition to introduction into ES cells or early embryos, the constructs may also be introduced by administration, e.g. in suitable vehicles or vectors, directly into the desired tissue of the whole organisms.

5           The multimerizing ligands useful for triggering the expression of the blocking gene including in the practice of this invention are capable of binding to two or more chimeric responder proteins containing such receptor domains. The multimerizing ligand may bind to the chimeras in either order or simultaneously, preferably with a Kd value below about  $10^{-6}$ , more preferably  
10 below about  $10^{-7}$ , even more preferably below about  $10^{-8}$ , and in some embodiments below about  $10^{-9}$  M. The ligand preferably is a non-protein and has a molecular weight of less than about 5 kDa. Even more preferably, the multimerizing ligand has a molecular weight of less than about 2 Kda, and even more preferably, less than 1500 Da. The receptor domains of the  
15 chimeric proteins so multimerized may be the same or different. The chimeric proteins are capable of initiating expression of the blocking gene in the host cell upon exposure to the ligand, following multimerization of the chimeras. Thus, transcription of the blocking gene or the elimination effector gene is activated in genetically engineered cells of this invention following exposure of  
20 the cells to a ligand capable of multimerizing the chimeras. Said differently, genetically engineered cells of this invention contain chimeric proteins as described above and are responsive to the presence of a ligand which is capable of multimerizing those chimera. That responsiveness is manifested by the initiation of expression of the blocking gene and by blockade of expression of  
25 the target gene or blocking a biological effect of the target gene product. Where regulatable elimination of a gene is desired, responsiveness is manifested by the initiation of Cre expression, and where a floxed target gene is also present, by the elimination of that target gene.

30           The encoded chimeric responder protein may further comprise an intracellular targeting domain capable of directing the chimeric protein to a desired cellular compartment. The targeting domain can be a secretory leader sequence, a membrane spanning domain, a membrane binding domain or a

sequence directing the protein to associate with vesicles or with the nucleus, for instance.

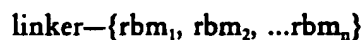
The action domains of the chimeric proteins may be selected from any of the proteins or protein domains (preferably of the species of the desired host cells or organism) which upon multimerization are capable of activating transcription of a gene, the blocking gene or the regulatable elimination effector gene, in our system, under the transcriptional control of a cognate control element. For instance, the action domain of the chimeric responder protein molecules may comprise a protein domain such as a CD3 $\zeta$  (zeta subunit) which is capable, upon exposure to the ligand and subsequent multimerization, of initiating a detectable intracellular signal leading to transcriptional activation via the IL-2 promoter. Alternatively, there may be a series of responder proteins, in which one responder protein contains as its action domain, a DNA-binding protein such as GAL4, while another contains as its action domain a transcriptional activation domain such as VP16. Heterodimerization of such responder proteins to form a GAL4-VP16 dimer activates the transcription of the blocking genes or the elimination effector gene under the transcriptional control of elements to which the heterodimerized responder proteins can bind. Numerous other examples are provided herein. In such examples, multimerization activates transcription of a blocking gene or an elimination effector gene under the transcriptional control of a transcriptional control element enhancer and/or promoter elements and the like, which is responsive to the multimerization event.

This invention further encompasses DNA vectors containing the various constructs described herein, whether for introduction into host cells in tissue culture, for introduction into embryos or for administration to whole organisms for the introduction of the constructs into cells in vivo. In either case the construct may be introduced episomally or for chromosomal integration. The vector may be a viral vector, including for example, an adeno-, adeno associated or retroviral vector. The constructs or vectors containing them may also contain selectable markers permitting selection of transfectants containing the construct.

This invention further encompasses a chimeric protein encoded by any of our DNA constructs, as well as cells containing and/or expressing them, including prokaryotic and eucaryotic cells and in particular, yeast, worm, insect, mouse or other rodent, and other mammalian cells, including human  
5 cells, of various types and lineages, whether frozen or in active growth, whether in culture or in a whole organism containing them.

This invention provides cells, preferably mammalian cells, which contain one or more recombinant DNA constructs encoding a responder protein, or series of responder proteins, and a blocking gene construct capable  
10 of expressing a blocking gene in response to multimerization of the responder protein(s). Additionally, it provides cells which contain one or more recombinant DNA constructs encoding a responder protein, or series of responder proteins, a Cre construct capable of expressing a Cre gene in response to multimerization of the responder protein(s) and optionally, a  
15 target gene construct comprising a target gene flanked by loxP sequences which is susceptible to deletion in the presence of Cre. The invention also provides cells which contain recombinant DNA constructs encoding proteins whose expression is capable of leading to regulated apoptosis of the cells containing the construct.

20 The multimerizing ligands are molecules capable of binding to two or more chimeric responder protein molecules of this invention to form a multimer thereof, and have the formula:



wherein n is an integer from 2 to about 5,  $\text{rbm}_{(1)}, \text{rbm}_{(n)}$  are receptor binding  
25 moieties which may be the same or different and which are capable of binding to the chimeric protein(s). The rbm moieties are covalently attached to a linker moiety which is a bi- or multi-functional molecule capable of being covalently linked ("—") to two or more rbm moieties. Preferably the ligand has a molecular weight of less than about 5 Kda and is not a protein. Examples  
30 of such ligands include those in which the rbm moieties are the same or

different and comprise an FK506-type moiety, a cyclosporin-type moiety, a steroid or tetracycline. Cyclosporin-type moieties include cyclosporin and derivatives thereof which are capable of binding to a cyclophilin, naturally occurring or modified, preferably with a  $K_d$  value below about  $10^{-6}$  M. In some embodiments it is preferred that the ligand bind to a naturally occurring receptor with a  $K_d$  value greater than about  $10^{-6}$  M and more preferably greater than about  $10^{-5}$  M. Illustrative ligands of this invention are those in which at least one rbm comprises a molecule of FK506, FK520, rapamycin or a derivative thereof modified at C9, C10 or both, which ligands bind to a modified receptor or chimeric molecule containing a modified receptor domain with a  $K_d$  value at least one, and preferably 2, and more preferably 3 and even more preferably 4 or 5 or more orders of magnitude less than their  $K_d$  values with respect to a naturally occurring receptor protein. Linker moieties are also described in detail later, but for the sake of illustration, include such moieties as a C2-C20 alkylene, C4-C18 azalkylene, C6-C24 N-alkylene azalkylene, C6-C18 arylene, C8-C24 ardiakylene or C8-C36 bis-carboxamido alkylene moiety. See also U.S. Patent Application Serial No. 08/481,941 entitled "Rapamycin Regulation of Biological Events", filed on June 7, 1995 and U.S. Patent Application Serial No. 08/292,598, entitled "New Multimerizing Agents," filed 18 August 1994, the contents of which are hereby incorporated by reference.

The monomeric rbm's of this invention, as well as compounds containing sole copies of an rbm, which are capable of binding to our chimeric proteins but not effecting dimerization or higher order multimerization thereof (in view of the monomeric nature of the individual rbm) are multimerization antagonists.

This invention thus provides materials and methods for selectively obstructing the expression or effects of a target gene in engineered cells in response to the presence of a multimerizing ligand which is added to the culture medium or administered to the whole organism. The invention further provides materials and methods for selectively eliminating a target gene in engineered cells in response to the presence of a multimerizing ligand which

is added to the culture medium or administered to the whole organism, as the case may be. The invention further provides materials and methods for selectively ablating cells in response to the addition of an oligomerizing ligand.

The methods involve providing cells of this invention, or an organism  
5 containing such cells, which contain and are capable of expressing (a) one or more DNA constructs encoding one or more chimeric proteins capable, following multimerization, of activating transcription of a blocking gene; (b) a blocking gene under the transcriptional regulation of an element responsive to multimers of the chimeric proteins. The method thus involves exposing the  
10 cells to a multimerization ligand capable of binding to the chimeric protein in an amount effective to result in detectable expression of the blocking gene.

The method also involves providing cells of this invention, or an organism containing such cells, which contain and are capable of expressing (a)  
15 one or more DNA constructs encoding one or more chimeric proteins capable, following multimerization, of activating transcription of a gene encoding Cre; (b) a gene encoding Cre which is under the transcriptional regulation of an element responsive to multimers of the chimeric proteins; and (c) a floxed target gene which is susceptible to recombination and elimination in the presence of Cre. The method thus involves exposing the cells to a  
20 multimerization ligand capable of binding to the chimeric protein in an amount effective to result in detectable expression of the Cre gene.

In cases in which the cells are growing in culture, exposure to the ligand is effected by adding the ligand to the culture medium. In cases in which the cells are present within a host organism, exposing them to the  
25 ligand is effected by administering the ligand to the host organism. For instance, in cases in which the host organism is an animal, in particular, a mammal the ligand is administered to the host animal by oral, buccal, sublingual, transdermal, subcutaneous, intramuscular, intravenous, intra-joint or inhalation administration in an appropriate vehicle therefor.

30 Where the target gene is essential for normal functioning in the healthy cell or animal, obstructing the expression of the gene, the effects of the gene product or eliminating the gene entirely, using the materials and methods of

this invention, provides cellular and animal models for corresponding disease states. For example, where the target gene is the gene for erythropoietin, its obstruction or elimination provides a model for the study of anemia and potential treatments for it. Targeting genes for nerve growth factors or myelinating proteins provides models of neural degenerative diseases; for bone morphogenic factors, of osteoporosis; for thrombopoietin, of thrombocytopenia; for antigen receptors or immune cell signaling proteins, of immunodeficiencies; for tyrosine hydroxylase, of Parkinson's disease; etc. See Table 1.

10           This invention further encompasses pharmaceutical or veterinary compositions for obstructing the expression or effects of a gene or eliminating a gene from genetically engineered cells of this invention, including eliminating the engineered cells themselves from animal tissue or from a subject containing such engineered cells. Such pharmaceutical or veterinary compositions  
15           comprise a multimerization ligand of this invention in admixture with a pharmaceutically or veterinarily acceptable carrier and optionally with one or more acceptable excipients. The multimerization ligand can be a homo-multimerization reagent or a hetero-multimerization reagent so long as it is capable of binding to a chimeric responder protein(s) of this invention,  
20           triggering expression of the blocking gene or triggering Cre expression in engineered cells of this invention. Likewise, this invention further encompasses a pharmaceutical or veterinary composition comprising a multimerization antagonist of this invention in admixture with a pharmaceutically acceptable carrier and optionally with one or more  
25           pharmaceutically or veterinarily acceptable excipients for preventing or reducing, in whole or part, the level of multimerization of chimeric responder proteins in engineered cells of this invention, in cell culture or in a subject, and thus for preventing or reversing the activation of transcription of the blocking gene in the relevant cells. Thus, the use of the multimerization  
30           reagents and of the multimerization antagonist reagents to prepare pharmaceutical or veterinary compositions is encompassed by this invention.

          This invention also offers a method for providing a host organism,



preferably an animal, and in many cases a mammal, susceptible to regulatable obstruction of a target gene in response to a multimerization ligand of this invention. The method involves introducing into the organism cells which have been engineered *ex vivo* in accordance with this invention, i.e. containing  
5 a DNA construct encoding a chimeric protein hereof, and so forth. Alternatively, one can introduce the DNA constructs of this invention into a host organism, e.g. mammal or embryo thereof, under conditions permitting transfection of one or more cells of the host mammal *in vivo*.

We further provide kits for producing cells susceptible to ligand-  
10 regulated obstruction of a target gene. One such kit contains at least one DNA construct encoding at least one of our chimeric responder proteins, comprising at least one receptor domain and at least one action domain (as described elsewhere). In one embodiment the DNA construct contains a conventional polylinker to provide the practitioner a site for the incorporation  
15 of cell-type specific expression control element(s), such as promoter and/or enhancer elements, to provide for cell-type or tissue-specific expression of one or more of the chimeras. The kit may contain a quantity of a ligand of this invention capable of multimerizing the chimeric protein molecules encoded by the DNA constructs of the kit, and may contain in addition a quantity of a  
20 multimerization antagonist, e.g. monomeric ligand reagent. Where a sole chimeric protein is encoded by the construct(s), the multimerization ligand is a homo-multimerization ligand. Where more than one such chimeric protein is encoded, a hetero-multimerization ligand may be included. The kit may further contain a blocking gene construct linked to a transcription control  
25 element responsive to multimerization of the chimeric responder protein molecules. Alternatively, the kit may contain a Cre construct linked to a transcription control element responsive to multimerization of the chimeric responder protein molecules and/or a target gene construct containing the target gene flanked by loxP sequence. The kit may also contain at least one  
30 DNA construct encoding a primary chimeric protein containing at least one receptor domain and one action domain where one action domain may be the cytoplasmic domain of Fas or of a TNF receptor as described below. The

DNA constructs will preferably be associated with one or more selectable markers for convenient selection of transfectants, as well as other conventional vector elements useful for replication in prokaryotes, for expression in eukaryotes, and the like. The selection markers may be the same or different for each different DNA construct, permitting the selection of cells which contain various combinations of such DNA construct(s).

For example, one kit of this invention contains vectors comprising a first DNA construct encoding a first chimeric responder protein containing at least one receptor domain (capable of binding to a selected ligand) fused to a transcriptional activator domain; a second DNA construct encoding a second chimeric responder protein containing at least one receptor domain fused to a DNA binding domain; and optionally, a third DNA construct encoding a blocking gene under the transcriptional control of an element responsive to the multimerization of the first and second chimeric responder proteins. Alternatively, the blocking gene construct may contain a cloning site, for example, a polylinker sequence, in place of a pre-selected blocking gene to permit the practitioner to insert any desired blocking gene.

The kit may also contain a DNA construct encoding Cre under the transcriptional control of an element responsive to the multimerization of the first and second chimeric responder proteins and optionally, a DNA construct encoding a target gene flanked by the loxP sequence, permitting deletion of the target gene in the presence of Cre. Alternatively, the fifth DNA construct may contain a cloning site in place of a target gene to permit the practitioner to insert any desired target gene.

Other kits of this invention may contain one, two, or more DNA constructs encoding chimeric proteins in which one or more of the constructs contain a cloning site in place of an action domain, such as the transcriptional initiation signal generator, transcriptional activator, the DNA binding protein, or other domains, permitting the user to insert whichever action domain s/he wishes. Such a kit may optionally include other elements as described above, such as a DNA construct for a target gene under responsive expression control, multimerization ligand, antagonist, etc.

Any of the kits may also contain positive control cells which were stably transformed with constructs of this invention such that they express a reporter gene such CAT (chloramphenicol transferase), beta-galactosidase or any other conveniently detectable gene product, in response to exposure of the cells to ligand. Reagents for detecting and/or quantifying the expression of the reporter gene may also be provided.

### Brief Description Of The Figures

Figure 1 is a diagram of the plasmid pSXNeo/IL2 (IL2-SX). In NF-AT-SX, the *HindIII*-*Clal* DNA fragment from IL2-SX containing the IL2 enhancer/promoter, is replaced by a minimal IL-2 promoter, which confers basal transcription, and an inducible element containing three tandem NFAT-binding sites. (These constructs are described in further detail below.)

Figure 2 is a flow diagram illustrating the preparation of the intracellular signaling chimera plasmids p#MXFn and p#MFnZ, where n indicates the number of binding domains.

Figs. 3A and 3B are a flow diagram of the preparation of the extracellular signalling chimera plasmid p#1FK3/pBJ5.

Figs. 4A, 4B and 4C are sequences of the primers used in the constructions of the plasmids employed in the subject invention.

Figure 5 is a chart of the response of reporter constructs having different enhancer groups to reaction of the receptor TAC/CD3 $\zeta$  with a ligand.

Figure 6 is a chart of the activity of various ligands with the TAg Jurkat cells described in Example 1. For Figure 6B, see also Spencer et al., Science 262,1019, Fig 3 and caption, esp. 3B on p. 1020 therein.

Figure 7 is a chart of the activity of the ligand FK1012A (8, Figure 9B) with the extracellular receptor 1FK3 (FKBPx3/CD3 $\zeta$ ).

Figure 8 is a chart of the activation of an NFAT reporter via signalling through a myristoylated CD3 $\zeta$ /FKBP12 chimera.

Figs. 9A and 9B are the chemical structures of the allyl-linked FK506 variants and the cyclohexyl-linked FK506 variants, respectively.

Figure 10 is a flow diagram of the synthesis of derivatives of FK520.

Figs. 11 A and B are a flow diagram of a synthesis of derivatives of FK520

and chemical structures of FK520, where the bottom structures are designed to bind to mutant FKBP12.

Figure 12 is a diagrammatic depiction of mutant FKBP with a modified FK520 in the putative cleft.

5        Figure 13 is a flow diagram of the synthesis of heterodimers of FK520 and cyclosporin.

Figure 14 is a schematic representation of the oligomerization of chimeric proteins, illustrated by chimeric proteins containing an immunophilin moiety as the receptor domain.

10       Figure 15 depicts ligand-mediated oligomerization of chimeric proteins, showing schematically the triggering of a transcriptional initiation signal.

Figure 16 depicts synthetic schemes for HED and HOD reagents based on FK506-type moieties.

Figure 17 depicts the synthesis of (CsA)<sub>2</sub> beginning with CsA.

15       Figure 18 is an overview of the fusion cDNA construct and protein MZF3E.

Figure 19 shows FK1012-induced cell death of the Jurkat T-cell line transfected with a myristoylated Fas-FKBP12 fusion protein (MFF3E), as indicated by the decreased transcriptional activity of the cells.

20       Figure 20A is an analysis of cyclophilin-Fas (and Fas-cyclophilin) fusion constructs in the transient transfection assay. MC3FE was shown to be the most effective in this series.

Figure 20B depicts Immunophilin-Fas antigen chimeras and results of transient expression experiments in Jurkat T cells stably transformed with large T-antigen. Myr: the myristylation sequence taken from pp60<sup>c-src</sup> encoding residues 1-14 (Wilson et al., *Mol & Cell Biol* 9 4 (1989): 1536-44); FKBP: human FKBP12; CypC: murine cyclophilin C sequence encoding residues 36-212 (Freidman et al., *Cell* 66 4 (1991): 799-806); Fas: intracellular domain of human Fas antigen encoding residues 179-319 (Oehm et al., *J. Biol. Chem.* 267 15 (1992): 10709-15).

30       Cells were electroporated with a plasmid encoding a secreted alkaline phosphatase reporter gene under the control of 3 tandem AP1 promoters along with a six fold molar excess of the immunophilin fusion construct. After 24 h (hours) the cells were stimulated with PMA (50ng/mL), which stimulates the synthesis of the

reporter gene, and (CsA)2. At 48 hours the cells were assayed for reporter gene activity. Western blots were performed at 24 hours using anti-HA epitope antibodies.

Figure 21 depicts the synthesis of modified FK506 type compounds.

## 5 I. Discussion

This invention provides chimeric proteins, organic molecules for multimerizing the chimeric proteins and a system for using them. The fused chimeric proteins have a binding domain for binding to the multimerizing molecule and an action domain, which can effectuate a physiological action or cellular process. Preferably the multimerizing molecule is a small organic molecule. The physiological action or cellular process effectuated by multimerization of the chimeric proteins is generally transcription of a gene encoding a regulatable blocking gene. This gene may be a "blocking" gene, which encodes a blocking factor such as an anti-sense message complementary to, and capable of interfering with transcription of a target gene or a ribozyme, which is capable of preventing the expression of a target gene. Alternatively, the blocking gene may encode an antibody moiety, such as a single-chain antibody, which is capable of binding to and preferably neutralizing or blocking a biological effect of the gene product of the target gene. The blocking gene also may encode a dominant negative gene product capable of blocking the effect of a target gene product, or it may encode a protein whose action will lead to the elimination of the target gene entirely. An example of the latter type would be the gene encoding the protein Cre, which produces Cre recombinase and whose expression leads to elimination of a target gene appropriately flanked by "loxP" sequences in the host cells. Alternatively, the constructs will encode gene products such as the cytoplasmic domain of the Fas antigen or TNF receptor, which allow the cells containing such constructs to be readily eliminated through apoptosis.

The basic concept of ligand-mediated multimerization is illustrated in figure 14. Divalent ligands which can function as heterodimerization, or hetero-multimerization, ("HED") agents and homodimerization, or homo-multimerization, ("HOD") agents are depicted as dumbbell-shaped structures.

The terms "homodimerization" and "homo-multimerization" refer to the

association of like components to form dimers or multimers, which may be linked, as shown in figure 14, by the multivalent ligands of this invention. The terms "heterodimerization" and "hetero-multimerization" refer to the association of dissimilar components to form dimers or higher order multimers. Homo-  
5 multimers thus comprise an association of multiple copies of a particular component, while hetero-multimers comprise an association of copies of different components. "Multimerization", "multimerize" and "multimer", as the terms are used herein, with or without prefixes, are intended to encompass "dimerization", "dimerize" and "dimer", absent an explicit indication to the contrary.

10 Also depicted in Figure 14 and in Figure 8 of Spencer et al., are fusion, or chimeric, protein molecules containing an action domain and one or more receptor domains that can bind to the multimerization ligands. Intracellular chimeric proteins, *i.e.*, proteins which are intended to be located within the cells in which they are produced, will in some embodiments preferably,  
15 contain a cellular targeting sequence (e.g. including organelle targeting amino acid sequences). Binding of the ligand to the receptor domains leads to multimerization of the fusion proteins. Multimerization brings the action domains into close proximity with one another thus triggering activation of transcription of a gene which is under the transcriptional control of an  
20 element responsive to the multimerization.

Cellular processes which can be triggered by receptor multimerization include a change in state, such as a physical state, *e.g.* conformational change, change in binding partner, cell death, initiation of transcription, channel opening, ion release, *e.g.*  $\text{Ca}^{+2}$  *etc.* or a chemical state, such as an enzymatically  
25 catalyzed chemical reaction, *e.g.* acylation, methylation, hydrolysis, phosphorylation or dephosphorylation, change in redox state, rearrangement, or the like. Thus, any such process which can be triggered by ligand-mediated multimerization is included within the scope of this technology, although the primary focus here is activation of transcription, directly or indirectly, of a  
30 blocking gene, including a gene whose gene product can function to block expression of a selected target gene or the eliminate the target gene from the cell.

In a central feature of this invention, cells are modified so as to be responsive to ligand molecules which are capable of binding to, and thus multimerizing, the primary chimeras disclosed herein. Such engineered cells respond to the presence of the multimerizing ligand by activating transcription of a blocking gene via a transcriptional control element responsive to the multimerized chimeric protein molecules.

The modified cells are characterized by a genome containing (a) a genetic construct (or series thereof) encoding a primary chimeric protein (or series of primary chimeric proteins) of this invention, which permits ligand-regulated transcriptional activation of a blocking gene via a corresponding transcriptional control element, and (b) a blocking gene construct comprising a recombinant DNA sequence containing a blocking gene under the transcriptional control of a transcriptional control sequence which is activated by multimerization of the chimeric proteins. Where the regulated blocking gene encodes the protein Cre, the cells additionally contain (c) a target gene flanked by loxP DNA sequences, permitting recombination and elimination of the target gene in the presence of Cre protein.

Only a single construct in the first series will be required where a homomultimer, including homodimers, is involved in activating the transcription of a blocking gene, while two or more constructs are required where a heteromultimer is involved. The chimeric proteins encoded by the first series of constructs will be associated with actuation of gene transcription and will normally be directed to the surface membrane or the nucleus.

There are two main classes of genetic constructs encoding the chimeric proteins of this invention. In both cases the encoded chimeric proteins contain at least one action domain and at least one ligand binding domain. The two classes are described as follows: (1) constructs wherein a ligand-binding domain of the encoded chimera is either extracellular or intracellular, but an action domain is intracellular, such that ligand-mediated, homo- or hetero-multimerization of the chimeric protein molecules induces a signal which results in a series of events resulting in transcriptional activation of the selected gene; (2) constructs wherein a ligand-binding domain and an action

domain of the encoded chimera is intranuclear, such that ligand-mediated, homo- or hetero-multimerization of the protein molecules induces initiation of transcription directly via complexation of multimers with the transcriptional initiation region of the selected blocking gene.

## 5 II. Transcription Regulation

The chimeric proteins encoded by the constructs of Groups (1) and (2), above, differ somewhat in their effects. Group (1) chimeras activate transcription indirectly and can have somewhat pleiotropic effects; that is, they may activate a number of wild-type genes in addition to the introduced  
10 blocking gene. Furthermore, the transcriptional activation following multimerization of group (1) chimeric proteins may be slower in onset than in the case of Group (2) chimeras. Group (2) constructs activate transcription more directly and in a manner more narrowly limited to the selected gene. The transcriptional activation in response to multimerization of Group (2)  
15 chimeric proteins is typically very rapid.

The chimeric protein contains a "binding" or "receptor" domain which is capable of binding to at least one ligand molecule. Since the multimerizing ligand is multivalent, in the sense that it contains more than one receptor-binding site, it can form dimers or higher order homo- or hetero-multimers  
20 with the chimeric proteins of this invention. The chimeric protein can have one or a plurality of binding sites, so that, for example, homomultimers can be formed with a divalent ligand.

The chimeric protein also contains an "action" domain capable, upon ligand-mediated multimerization of the chimeric protein molecules, of  
25 initiating transcription, whether directly or indirectly.

The chimeric proteins, whether of Group (1) or (2) will typically also contain an intracellular targeting domain comprising a sequence or component which directs the chimeric protein to the desired compartment, for example to the surface membrane in the case of Group (1), or the nucleus in the case of  
30 Group (2).

By way of illustration, a Group (1) chimeric protein may contain a



myristate moiety as an intracellular targeting domain, three FKBP12 moieties as receptor domains; and the action domain comprising a T cell receptor  $\zeta$  subunit. See for example, Spencer et al., *Science* (1993). Group (2) chimeric proteins generally comprise a series of at least two chimeras, where the action domains of one comprise DNA-binding domains, while the action domains of another comprise transcriptional activating domains. Multimerization of the Group (2) chimeras brings the DNA-binding domains and transcriptional activation domains in close proximity. Subsequent interaction of resulting multimers with the DNA sequence to which the DNA-binding domains bind results in the initiation of transcription of the gene associated with the responsive DNA element.

In either case, a gene encoding the desired anti-sense message, ribozyme, antibody moiety, dominant protein or protein capable of functioning in the elimination of a gene sequence, such as the protein Cre, must be provided in a manner such that it is under the transcriptional control of a DNA element responsive, directly or indirectly, to the addition of the multimerizing ligand, i.e., to multimerization of the Group (1) or Group (2) chimeric protein molecules. In the case of Group (1) chimeras the blocking gene is linked to a transcriptional regulatory sequence activated upon multimerization of the chimera's action domain. For example, where the action domain is a T-cell CD3 $\zeta$  subunit, the blocking gene may be linked to NFAT sequence. Where the desired regulatable gene encodes the protein Cre, a recombinant construct comprising a floxed target gene is provided to serve as the object of the Cre-mediated recombination to complete the overall system.

#### A. Surface Membrane Receptor

Group (1) chimeric proteins of this invention are typically associated with the surface membrane of the engineered cells. Addition of the multimerizing agent to cells containing such proteins results in the generation of a signal leading to transcription of one or more genes. The process involves a number of auxiliary proteins in a series of interactions culminating in the binding of transcription factors to promoter regions associated with the

selected gene(s) In cases in which the transcription factors bind to promoter regions associated with other genes, transcription is initiated there as well. A construct encoding a chimeric protein of this embodiment can encode a signal sequence which can be subject to processing and therefore may not be present in the mature chimeric protein. The chimeric protein will in any event comprise (a) a binding domain capable of binding a pre-determined ligand, (b) an optional (although in many embodiments, preferred) membrane binding element or domain which includes a transmembrane domain or an attached lipid for translocating the fused protein to the cell surface/membrane and retaining the protein bound to the cell surface membrane, and, (c) as the action domain, a cytoplasmic signal initiation domain. The cytoplasmic signal initiation domain is capable of initiating a signal which results in transcription of a gene having a recognition sequence for the initiated signal in the transcriptional initiation region.

The molecular portion of the chimeric protein which provides for binding to a membrane is also referred to as the "retention domain". Suitable retention domains include a moiety which binds directly to the lipid layer of the membrane, such as through lipid participation in the membrane or extending through the membrane, or the like. In such cases the protein becomes translocated to and bound to the membrane, particularly the cellular membrane, as depicted in figure 15. Also see figure 8 of Spencer et al.

#### B. Nuclear Transcription Factors

Group (2) chimeric proteins may contain a cellular targeting sequence which provides for the protein to be translocated to the nucleus. This "signal consensus" sequence has a plurality of basic amino acids, referred to as a bipartite basic repeat as reviewed in Garcia-Bustos et al., *Biochimica et Biophysica Acta* (1991) 1071, 83-101. This sequence can appear in any portion of the molecule internal or proximal to the N- or C-terminus and results in the chimeric protein being inside the nucleus. One embodiment of this invention involves at least two such Group (2) chimeric proteins: (1) one having an action domain which binds to the DNA of the transcription

initiation region associated with the blocking gene in the blocking gene construct and (2) a different chimeric protein containing as an action domain, a transcriptional activation domain capable, in association with the DNA binding domain of the first chimeric protein, of initiating transcription of the blocking gene construct. The two action domains or transcription factors can be derived from the same or different protein molecules.

The transcription factors can be endogenous or exogenous to the cellular host. If the transcription factors are exogenous, but functional within the host and can cooperate with the endogenous RNA polymerase, rather than requiring an exogenous RNA polymerase, for which a gene could be introduced, then an exogenous promoter element functional with the fused transcription factors can be provided within the blocking gene construct for regulating transcription of the blocking gene. By this means the initiation of transcription can be restricted to the blocking gene associated with the heterologous promoter region.

A large number of transcription factors are known which require two subunits for activity. Alternatively, in cases where a single transcription factor can be divided into two separate functional domains (e.g. a transcriptional activator domain and a DNA-binding domain), so that each domain is inactive by itself, but when brought together in close proximity, transcriptional activity is restored. Transcription factors which can be used include yeast GAL4, which can be divided into two domains as described by Fields and Song, *supra*. The authors use a fusion of GAL4(1-147)-SNF1 and SNF4-GAL4(768-881), where the SNF1 and -4 may be replaced by the subject binding proteins as binding domains. Combinations of GAL4 and VP16 or HNF-1 can be employed. Other transcription factors are members of the Jun, Fos, and ATF/CreB families, Oct1, Sp1, HNF-3, the steroid receptor superfamily, and the like.

As an alternative to using the combination of a DNA binding domain and a naturally occurring activation domain or modified form thereof, the activation domain may be replaced by one of the binding proteins associated with bridging between a transcriptional activation domain and an RNA

polymerase, including, but not limited to RNA polymerase II. These proteins include the proteins referred to as TAF's (transcriptional activation factors), the TFII proteins, particularly B and D, or the like. Thus, one can use any one or combination of proteins, for example, fused proteins or binding motifs thereof, which serve in the bridge between the DNA binding protein and RNA polymerase and provide for initiation of transcription. Preferably, the protein closest to the RNA polymerase will be employed in conjunction with the DNA binding domain to provide for initiation of transcription. If desired, the subject constructs can provide for three or more, usually not more than about 4, proteins to be brought together to provide the transcription initiation complex.

Rather than have a transcriptional activation domain as an action domain, an inactivation domain, such as *ssn-6/TUP-1* or Krüppel-family suppressor domain, can be employed. In this manner, regulation results in turning off the transcription of a gene which is constitutively expressed. For example, in the case of gene therapy one can provide for constitutive expression of a hormone, such as growth hormone, blood proteins, immunoglobulins, etc. By employing constructs encoding one chimeric protein containing a DNA binding domain joined to a ligand binding domain and another chimeric protein containing an inactivation domain joined to a ligand binding domain, the expression of the gene can be inhibited via ligand-mediated multimerization.

Constructs encoding a chimeric protein containing *inter alia* a ligand-binding domain fused to a transcriptional activating domain or subunit, or transcriptional inactivating domain or DNA-binding domain are designed and assembled in the same manner as described for the other constructs. Frequently, the N-terminus of the transcription factor will be bound to the C-terminus of the ligand-binding domain, although in some cases the reverse will be true, for example, where two individual domains of a single transcription factor are divided between two different chimeras.

### III. Components & Their Functions in the Chimeric Proteins

There is a considerable amount of flexibility in the selection of component domains and their incorporation into the design of chimeric proteins of this invention. For chimeric proteins intended for association with the surface membrane, if the ligand-binding domain is extracellular, the chimeric protein can be designed to contain an extracellular domain selected from a variety of surface membrane proteins. Similarly, various cytoplasmic or intracellular domains of the surface membrane proteins which are able to transduce a signal can be employed, depending on which endogenous genes are regulated by the cytoplasmic portion. Where the chimeric protein is to be internal to the cell, internal to the surface membrane protein or associated with an organelle, such as the nucleus or a cytoplasmic vesicle, the ligand-binding domain protein will preferably be one which can bind molecules able to cross the surface membrane or other membrane, as appropriate. These binding domains will generally bind to multivalent ligands comprising naturally occurring or synthetic ligand moieties, which are preferably not nucleic acids or peptidic.

#### A. Cytoplasmic Domains for Group (1) Chimeras

A chimeric protein of Group (1) can contain as an action domain, a cytoplasmic domain from one of the various cell surface membrane receptors or variants thereof for which a corresponding recognition sequence is known or available and which is capable of initiating transcription in response to multimerization of the chimeric protein. Such recognition sequences include those associated with a gene responsive to transcriptional activation triggered by such a receptor. Mutant receptors of interest will dissociate transcriptional activation of a selected gene from activation of genes which can be associated with harmful side effects, such as deregulated cell growth or inappropriate release of cytokines. The receptor-associated cytoplasmic domains of particular interest will have the following characteristics: receptor activation leads to initiation of transcription for relatively few (desirably fewer than 100) and generally innocuous genes in the cellular host; the other factors necessary for transcription initiated by receptor activation are present in the cellular host;

genes which are activated other than the selected gene, will not affect the intended purpose for which these cells are to be used; multimerization of the cytoplasmic domain or other available mechanism results in signal initiation; and joining of the cytoplasmic domain to a desired ligand-binding domain will not interfere with signalling. A number of different cytoplasmic domains are known. Many of these domains are tyrosine kinases or are complexed with tyrosine kinases, and include CD3 $\zeta$ , IL-2R, and IL-3R, among others. (See Cantley, *et al.*, *Cell* (1991) 64, 281.) Tyrosine kinase receptors, which are activated by cross-linking, or dimerization include subclass I: EGF-R, ATR2/neu, HER2/neu, HER3/c-erbB-3, Xmrk; subclass II: insulin-R, IGF-1-R (insulin-like growth factor receptor), IRR; subclass III: PDGF-R-A, PDGF-R-B, CSF-1-R (M-CSF/c-Fms), c-kit, STK-1/Flk-2; and subclass IV: FGF-R, flg [acidic FGF], bek [basic FGF]; neurotrophic tyrosine kinases: Trk family, includes NGF-R, Ror1,2. (Based on nomenclature first proposed by Yarden and Ulrich, *Ann. Rev. Biochem.* (1988) 57, 443.) Receptors which associate with tyrosine kinases upon cross-linking include the CD3  $\zeta$ -family: CD3  $\zeta$  and CD3  $\eta$ , which are found primarily in T cells, and associate with Fyn;  $\beta$  and  $\gamma$  chains of Fc $\epsilon$ RI, which are found primarily in mast cells and basophils;  $\gamma$  chain of Fc $\gamma$ RIII/CD16, which is found primarily in macrophages, neutrophils and natural killer cells; CD3 $\gamma$ ,  $\delta$ , and  $\epsilon$ , which are found primarily in T cells; Ig- $\alpha$ /MB-1 and Ig- $\beta$ /B29, which are found primarily in B cells. Many cytokine and growth factor receptors associate with common  $\beta$  subunits which interact with tyrosine kinases and/or other signalling molecules and which can be used as cytoplasmic domains in chimeric proteins of this invention. These include (1) the common  $\beta$  subunit shared by the GM-CSF, IL-3 and IL-5 receptors; (2) the  $\beta$  chain gp130 associated with the IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M, and IL-11 receptors; (3) the IL-2 receptor  $\gamma$  subunit associated also with receptors for IL-4, IL-7 and IL-13, and possibly IL-9; and (4) the  $\beta$  chain of the IL-2 receptor which is homologous to the cytoplasmic domain of the G-CSF receptor.

The interferon family of receptors which include interferons  $\alpha/\beta$  and  $\gamma$

(which can activate one or more members of the JAK, Tyk family of tyrosine kinases) as well as the receptors for growth hormone, erythropoietin and prolactin (which also can activate JAK2) can also be used as sources for cytoplasmic domains.

5        Other sources of cytoplasmic domains include the TGF- $\beta$  family of cell surface receptors. (Reviewed by Kingsley, D., *Genes and Development* 1994 8 133.) This family of receptors contains serine/threonine kinase activity in their cytoplasmic domains, which are believed to be activated by crosslinking.

10        The tyrosine kinases associated with activation and inactivation of transcription factors are of particular interest in providing specific pathways which can be controlled and can be used to initiate or inhibit expression of an exogenous gene.

15        The following table provides a number of receptors and characteristics associated with the receptor and their nuclear response elements that activate transcription of genes. The list is intended to provide exemplary systems (rather than an exhaustive list) for use in the subject invention.

20        In many situations mutated cytoplasmic domains can be obtained where the signal which is transduced may vary from the wild type, resulting in a restricted or different pathway as compared to the wild-type pathway(s). For example, in the case of growth factors, such as EGF and FGF, mutations have been reported where the signal is uncoupled from cell growth but is still maintained with c-fos (Peters, *et al.*, *Nature* (1992) 358, 678).

25        The tyrosine kinase receptors can be found on a wide variety of cells throughout the body. In contrast, the CD3 $\zeta$ -family, the Ig family and the lymphokine  $\beta$ -chain receptor family are found primarily on hematopoietic cells, particularly T-cells, B-cells, mast cells, basophils, macrophages, neutrophils, and natural killer cells. The signals required for NF-AT transcription come primarily from the  $\zeta$  (zeta) chain of the antigen receptor and to a lesser extent CD3 $\gamma$ ,  $\delta$ ,  $\epsilon$ .

Table 2

	Ligand	DNA Element	Binding Factors	Gene	Reference
	Insulin and others	cAMP responsive element (cre)	LRFI	jun-B many genes	Mol. Cell Biol. (1992), 12, 4654 PNAS, 83, 3439
5	PDGF, FGF, TGF and others	SRE	SRF/SREBP	c-fos	Mol. Cell Biol. (1992), 12, 4769
	EGF	VL30 RSRF		RVL-3 virus c-jun	Mol. Cell. Biol. (1992), 12, 2793 do. (1992), 12, 4472
	IFN- $\alpha$	ISRE	ISGF-3		Gene Dev. (1989) 3, 1362
10	IFN- $\gamma$	GAS	GAF	GBP	Mol. Cell. Biol. (1991) 11, 182
	PMA and TCR		AP-1	many genes	Cell (1987) 49, 729-739
	TNF		NF $\kappa$ B	many genes	Cell (1990) 62, 1019-1029
	Antigen	ARRE-1	OAP/Oct-1	many genes	Mol. Cell. Biol. (1988) 8, 1715
15	Antigen	ARRE-2	NFAT	IL-2 enhancer	Science (1988) 241, 202

The cytoplasmic domain, as it exists naturally or as it may be truncated, modified or mutated, will be at least about 10, usually at least about 30 amino acids, more usually at least about 50 amino acids, and generally not more than about 400 amino acids, usually not more than about 200 amino acids. (See Romeo, *et al.*, *Cell* (1992) 68, 889-893.) While any species can be employed, the species endogenous to the host cell is usually preferred. However, in many cases, the cytoplasmic domain from a different species can be used effectively. Any of the above indicated cytoplasmic domains may be used, as well as others



which are presently known or may subsequently be discovered.

For the most part, the other chimeric proteins associated with transcription factors, will differ primarily in having a cellular targeting sequence which directs the chimeric protein to the internal side of the nuclear membrane and having transcription factors or portions thereof as the action domains. Usually, the transcription factor action domains can be divided into DNA binding domains and activation domains. One can provide for a DNA binding domain with one or more ligand binding domains and an activation domain with one or more ligand binding domains. In this way the DNA binding domain can be coupled to a plurality of binding domains and/or activation domains. Otherwise, the discussion for the chimeric proteins associated with the surface membrane for signal transduction is applicable to the chimeric proteins for direct binding to genomic DNA.

#### B. Cellular Targeting Domains

A signal peptide or sequence provides for transport of a chimeric protein to the cell surface membrane, where the same or other sequences can result in binding of the chimeric protein to the cell surface membrane. While there is a general motif of signal sequences, two or three N-terminal polar amino acids followed by about 15-20 primarily hydrophobic amino acids, the individual amino acids can be widely varied. Therefore, substantially any signal peptide can be employed which is functional in the host and may or may not be associated with one of the other domains of the chimeric protein. Normally, the signal peptide is processed and will not be retained in the mature chimeric protein. The sequence encoding the signal peptide is at the 5'-end of the coding sequence and will include the initiation methionine codon.

The choice of membrane retention domain is not critical to this invention, since it is found that such membrane retention domains are substantially fungible and there is no critical amino acid required for binding or bonding to another membrane region for activation. Thus, the membrane retention domain can be isolated from any convenient surface membrane or cytoplasmic protein, whether endogenous to the host cell or not.

There are at least two different membrane retention domains: a transmembrane retention domain, which is an amino acid sequence which extends across the membrane; and a lipid membrane retention domain, which lipid associates with the lipids of the cell surface membrane.

5 For the most part, for ease of construction, the transmembrane domain of a cytoplasmic domain or a receptor domain can be employed, which may tend to simplify the construction of the fused protein. However, for the lipid membrane retention domain, the processing signal will usually be added at the 5' end of the coding sequence for N-terminal binding to the membrane and, 10 proximal to the 3' end for C-terminal binding. The lipid membrane retention domain will have a lipid of from about 12 to 24 carbon atoms, particularly 14 carbon atoms, more particularly myristoyl, joined to glycine. The signal sequence for the lipid binding domain is an N-terminal sequence and can be varied widely, usually having glycine at residue 2 and lysine or arginine at 15 residue 7 (Kaplan, *et al.*, *Mol. Cell. Biol.* (1988) 8, 2435). Peptide sequences involving post-translational processing to provide for lipid membrane binding are described by Carr, *et al.*, *PNAS USA* (1988) 79, 6128; Aitken, *et al.*, *FEBS Lett.* (1982) 150, 314; Henderson, *et al.*, *PNAS USA* (1983) 80, 319; Schulz, *et al.*, *Virology* (1984), 123, 2131; Dellman, *et al.*, *Nature* (1985) 314, 374; and 20 reviewed in *Ann. Rev. of Biochem.* (1988) 57, 69. An amino acid sequence of interest includes the sequence M-G-S-S-K-S-K-P-K-D-P-S-Q-R. Various DNA sequences can be used to encode such sequence in the fused receptor protein.

Generally, the transmembrane domain will have from about 18-30 amino acids, more usually about 20-30 amino acids, where the central portion 25 will be primarily neutral, non-polar amino acids, and the termini of the domain will be polar amino acids, frequently charged amino acids, generally having about 1-2 charged, primarily basic amino acids at the termini of the transmembrane domain followed by a helical break residue, e.g. pro- or gly-.

### C. Tissue Specific Expression of the Chimeric Proteins

30 It will be preferred in certain embodiments, that the target gene be regulatably eliminated in a cell-specific or tissue-specific manner. To achieve

such specificity, one may render the expression of the chimeric proteins cell-type specific. Such specificity of expression may be achieved by linking one or more of the DNA sequences encoding the chimeric protein(s) to a cell-type specific transcriptional regulatory sequence (e.g. promoter/enhancer).

- 5 Numerous cell-type specific transcriptional regulatory sequences are known. Others may be obtained from genes which are expressed in a cell-specific manner.

For example, constructs for expressing the chimeric proteins may contain regulatory sequences derived from known genes for specific expression  
10 in selected tissues. Representative examples are tabulated below:

Tissue	Gene	Reference
lens	$\gamma$ 2-crystallin	Breitman, M.L., Clapoff, S., Rossant, J., Tsui, L.C., Golde, L.M., Maxwell, L.H., Bernstein, A., Genetic Ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice, <i>Science</i> 238 (1987) 1563-1565.
	$\alpha$ A-crystallin	Landel, C.P., Zhao, J., Bok, D., Evans, G.A., Lens-specific expression of a recombinant ricin induces developmental defects in the eyes of transgenic mice, <i>Genes Dev.</i> 2 (1988), 1168-1178.
		Kaur, S., Key, B., Stock, J., McNeish, J.D., Akeson, R., Potter, S.S., Targeted ablation of alpha-crystallin-synthesizing cells produces lens-deficient eyes in transgenic mice, <i>Development</i> 105 (1989) 613-619.

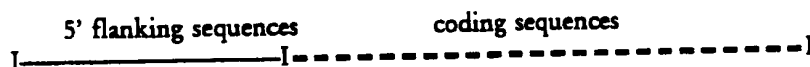
pituitary somatrophic cells	Growth hormone	Behringer, R.R., Mathews, L.S., Palmiter, R.D., Brinster, R.L., Dwarf mice produced by genetic ablation of growth hormone-expressing cells, <i>Genes Dev.</i> 2 (1988) 453-461.
pancreas	Insulin- Elastase - acinar cell specific	Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., MacDonald, R.J., Specific expression of an elastase-human growth fusion in pancreatic acinar cells of transgenic mice, <i>Nature</i> 311 (1985) 600-603.  Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxwell, F., Maxwell, I.H., Brinster, R.L., Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene, <i>Cell</i> 50 (1987) 435-443.
T cells	lck promoter	Chaffin, K.E., Beals, C.R., Wilkie, T.M., Forbush, K.A., Simon, M.I., Perlmutter, R.M., <i>EMBO Journal</i> 9 (1990) 3821-3829.
B cells	Immunoglobulin kappa light chain	Borelli, E., Heyman, R., Hsi, M., Evans, R.M., Targeting of an inducible toxic phenotype in animal cells, <i>Proc. Natl. Acad. Sci. USA</i> 85 (1988) 7572-7576.  Heyman, R.A., Borelli, E., Lesley, J., Anderson, D., Richmond, D.D., Baird, S.M., Hyman, R., Evans, R.M. Thymidine kinase obliteration: creation of transgenic mice with controlled immunodeficiencies, <i>Proc. Natl. Acad. Sci. USA</i> 86 (1989) 2698-2702.

Schwann cells	P <sub>0</sub> promoter	Messing, A., Behringer, R.R., Hammang, J.P. Palmiter, RD, Brinster, RL, Lemke, G., P <sub>0</sub> promoter directs expression of reporter and toxin genes to Schwann cells of transgenic mice, <i>Neuron</i> 8 (1992) 507-520.
	Myelin basic protein	Miskimins, R. Knapp, L., Dewey, M.J., Zhang, X. Cell and tissue-specific expression of a heterologous gene under control of the myelin basic protein gene promoter in transgenic mice, <i>Brain Res. Dev.</i> 65 (1992) 217-21.
spermatids	protamine	Breitman, M.L., Rombola, H., Maxwell, L.H., Klintworth, G.K., Bernstein, A., Genetic ablation in transgenic mice with attenuated diphtheria toxin A gene, <i>Mol. Cell. Biol.</i> 10 (1990) 474-479.
lung	Lung surfactant gene	Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., MacDonald, R.J., Specific expression of an elastase-human growth fusion in pancreatic acinar cells of transgenic mice, <i>Nature</i> 331 (1985) 600-603.
adipocyte P2		Ross, S.R., Braves, RA, Spiegelman, B.M., Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity, <i>Genes and Dev.</i> 7 (1993) 1318-24.
muscle	myosin light chain	Lee, K.J., Ross, R.S., Rockman, HA, Harris, AN, O'Brien, TX, van-Bilsen, M., Shubeita, HE, Kandolf, R., Brem, G., Prices et al., <i>J. Biol. Chem.</i> 267 (1992) 15875-85.

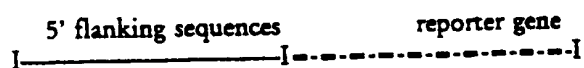
	Alpha actin	Muscat, G.E., Perry, S. , Prentice, H. Kedes, L. The human skeletal alpha-actin gene is regulated by a muscle-specific enhancer that binds three nuclear factors, <i>Gene Expression</i> 2 (1992) 111-26.
neurons	neurofilament proteins	Reeben, M. Halmekyto, M. Alhonen, L. Sinervirta, R. Saarma, M. Janne, J., Tissue-specific expression of rat light neurofilament promoter-driven reporter gene in transgenic mice, <i>BBRC</i> 192 (1993) 465-70.
liver	tyrosine aminotransferase, albumin, apolipoproteins	

#### Identification of tissue specific promoters

- 5 To identify the sequences that control the tissue- or cell-type specific expression of a gene, one isolates a genomic copy of the selected gene including sequences "upstream" from the exons that code for the protein.

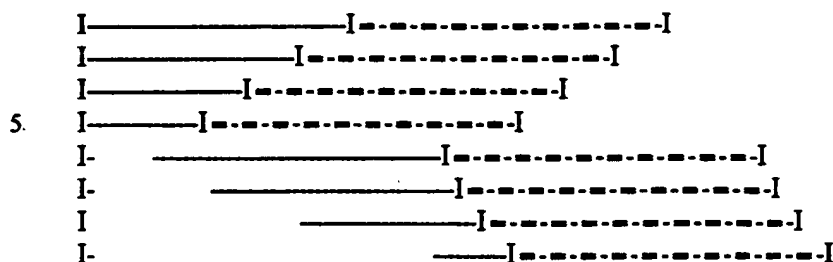


- 10 These upstream sequences are then usually fused to an easily detectable reporter gene like beta-galactosidase, in order to be able to follow the expression of the gene under the control of upstream regulatory sequences.



- 15 To establish which upstream sequences are necessary and sufficient to control gene expression in a cell-type specific manner, the complete upstream sequences are introduced into the cells of interest to determine whether the initial clone contains the control sequences. Reporter gene expressor is

monitored as evidence of expression.



- 10 If these sequences contain the necessary sequences for cell-type specific expression, deletions may be made in the 5' flanking sequences to determine which sequences are minimally required for cell-type specific expression. This can be done by making transgenic mice with each construct and monitoring beta-gal expression, or by first examining the expression in specific culture cells,
- 15 with comparison to expression in non-specific cultured cells.

Several successive rounds of deletion analysis normally pinpoint the minimal sequences required for tissue specific expression. Ultimately, these sequences are then introduced into transgenic mice to confirm that the expression is only detectable in the cells of interest.

#### 20 D. Ligand Binding Domain

- The ligand binding ("dimerization" or "receptor") domain of any of the chimeric proteins of this invention can be any convenient domain which binds to a natural or preferably, to a synthetic ligand. The location of the binding domain in the chimeric protein, as expressed and located within a cell, can be
- 25 internal or external to the cellular membrane, depending upon the nature of the chimeric protein and the choice of ligand. A wide variety of binding proteins are known, including receptors and binding proteins associated with the cytoplasmic regions indicated above. Binding proteins for which ligands are known or may be readily produced are of particular interest. These ligands are
- 30 preferably small organic molecules. The receptors or ligand binding domains include the FKBP's and cyclophilin receptors, the steroid receptors, the

tetracycline receptor, the other receptors indicated above, and the like, as well as "unnatural" receptors, which can be obtained from antibodies, particularly the heavy or light chain subunit, mutated sequences thereof, random amino acid sequences obtained by stochastic procedures, combinatorial syntheses, and the like. For the most part, the receptor domains will be at least about 50 amino acids, and fewer than about 350 amino acids, usually fewer than 200 amino acids, either as the natural domain or truncated active portion thereof. Preferably the binding domain will be small, usually less than 25 kDa, to allow efficient transfection in viral vectors, and will be monomeric, nonimmunogenic. Additionally, it should bind synthetically accessible, cell permeable, nontoxic ligands that can be configured for dimerization.

The receptor domain of the chimeric proteins expressed by a host cell can be intracellular or extracellular, depending upon the design of the construct encoding the chimeric protein and the availability of an appropriate ligand. For hydrophobic ligands, the binding domain can be on either side of the membrane, but for hydrophilic ligands, particularly protein ligands, the binding domain will usually be external to the cell membrane, unless there is a transport system for internalizing the ligand in a form in which it is available for binding. For an intracellular receptor, the construct can encode a signal peptide and transmembrane domain 5' or 3' of the receptor domain sequence or by having a lipid attachment signal sequence 5' or 3' of the receptor domain sequence. Where the receptor domain is between the signal peptide and the transmembrane domain, the receptor domain will be extracellular.

The portion of the construct encoding the receptor can be subjected to mutagenesis for a variety of reasons. The mutagenized protein can provide for higher binding affinity, allow for discrimination by the ligand of the naturally occurring receptor and the mutagenized receptor, provide opportunities to design a receptor-ligand pair, or the like. The change in the receptor can involve changes in amino acids known to be at the binding site, random mutagenesis using combinatorial techniques, where the codons for the amino acids associated with the binding site or other amino acids associated with conformational changes can be subject to mutagenesis by changing the codon(s)



for the particular amino acid, either with known changes or randomly, expressing the resulting proteins in an appropriate prokaryotic host and then screening the resulting proteins for binding. Illustrative of this situation is to modify FKBP12's Phe36 to Ala and/or Asp37 to Gly or Ala to accommodate a  
5 substituent at positions 9 or 10 of FK506 or FK520 or related ligands. In particular, mutant FKBP12 moieties which contain Val., Ala, Gly, Met or other small amino acids in place of one or more of Tyr26, Phe36, Asp37, Tyr82 and Phe99 are of particular interest as receptor domains for FK506-type and FK520-type ligands containing modifications at C9 and/or C10.

10 Antibody subunits, e.g. heavy or light chain, particularly fragments, more particularly all or part of the variable region, or fusions of heavy and light chain to create high-affinity binding, can be used as the binding domain. Antibodies can be prepared against haptenic molecules which are physiologically acceptable and the individual antibody subunits screened for  
15 binding affinity. The cDNA encoding the subunits can be isolated and modified by deletion of the constant region, portions of the variable region, mutagenesis of the variable region, or the like, to obtain a binding protein domain that has the appropriate affinity for the ligand. In this way, almost any physiologically acceptable haptenic compound can be employed as the ligand or  
20 to provide an epitope for the ligand. Instead of antibody units, natural receptors can be employed, where the binding domain is known and there is a useful ligand for binding.

The ability to employ in vitro mutagenesis or combinatorial modifications of sequences encoding proteins allows for the production of  
25 libraries of proteins which can be screened for binding affinity for different ligands. For example, one can totally randomize a sequence of 1 to 5, 10 or more codons, at one or more sites in a DNA sequence encoding a binding protein, make an expression construct and introduce the expression construct into a unicellular microorganism, and develop a library. One can then screen  
30 the library for binding affinity to one or desirably a plurality of ligands. The best affinity sequences which are compatible with the cells into which they would be introduced can then be used as the binding domain. The ligand

would be screened with the host cells to be used to determine the level of binding of the ligand to endogenous proteins. A binding profile could be defined by weighting the ratio of binding affinity to the mutagenized binding domain with the binding affinity to endogenous proteins. Those ligands which  
5 have the best binding profile could then be used as the ligand. Phage display techniques, as a non-limiting example, can be used in carrying out the foregoing.

#### E. Multimerization

The transduced signal will normally result from ligand-mediated  
10 multimerization of the chimeric protein molecules, i.e. as a result of multimerization following ligand binding, although other binding events, for example allosteric activation, can be employed to initiate a signal. The construct of the chimeric protein will vary as to the order of the various domains and the number of repeats of an individual domain. For the  
15 extracellular receptor domain in the 5'-3' direction of transcription, the construct will encode a protein comprising the signal peptide, the receptor domain, the transmembrane domain and the signal initiation domain, which last domain will be intracellular, either nuclear or cytoplasmic. However, where the receptor domain is intracellular, different orders may be employed,  
20 where the signal peptide can be followed by either the receptor or signal initiation domain, followed by the remaining domain, or with a plurality of receptor domains, the signal initiation domain can be sandwiched between receptor domains. Usually, the active site of the signal initiation domain will be internal to the sequence and not require a free carboxyl terminus. Either of  
25 the domains can be present in multiple copies, particularly the receptor domain, usually having not more than about 5 repeats, more usually not more than about 3 repeats.

For multimerizing the receptor, the ligand for the receptor domains of the chimeric surface membrane proteins will usually be multimeric in the sense  
30 that it will have at least two binding sites, each of which being capable of binding to a receptor domain. Desirably, the ligand will be a dimer or higher

order multimer, usually not greater than about tetrameric, of small synthetic organic molecules, the individual molecules typically being at least about 150 D and fewer than about 5 kD, usually fewer than about 3 kD. A variety of pairs of synthetic ligands and receptors can be employed. For example, in  
5   embodiments involving natural receptors, dimeric FK506 can be used with an FKBP receptor, dimerized cyclosporin A can be used with the cyclophilin receptor, dimerized estrogen with an estrogen receptor, dimerized glucocorticoids with a glucocorticoid receptor, dimerized tetracycline with the tetracycline receptor, dimerized vitamin D with the vitamin D receptor, and  
10   the like. Alternatively, higher orders of the ligands, e.g. trimeric can be used. For embodiments involving unnatural receptors, such as antibody subunits, modified antibody subunits or modified receptors and the like, any of a large variety of compounds can be used. A significant characteristic of these ligand units is that they bind the receptor with high affinity (preferably with a  $K_d \leq$   
15    $10^{-8}$  M) and they are able to be dimerized chemically.

The ligand can have different receptor binding molecules with different epitopes which are referred to as "HED" reagents, since they can mediate hetero-dimerization or hetero-multimerization of chimeric proteins having the same or different binding domains. For example, the ligand may comprise  
20   FK506 or an FK506-type moiety and a CsA or a cyclosporin type moiety. Both moieties are covalently attached to a common linker moiety. Such a ligand would be useful for mediating the multimerization of a first and second chimeric protein where the first chimeric protein contains a receptor domain such as an FKBP12 which is capable of binding to the FK506-type moiety and  
25   the second chimeric protein contains a receptor domain such as cyclophilin which is capable of binding to the cyclosporin A-type moiety.

#### IV. Cells

For applications in which one wishes to engineer all or substantially all cells of an organism, standard microinjection of embryos or use of ES cells is  
30   preferred. For other applications the cells may be procaryotic, but are preferably eucaryotic, including plant, yeast, worm, insect and mammalian.

Those cells may be mammalian cells, from any mammal of interest, particularly domesticated animals, such as horses, cows, pigs, dogs, cats, rats, mice and so forth. Among these species, various types of cells can be involved, such as osteoclasts, osteoblasts, neuronal, hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle, spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, pancreatic, gastrointestinal, pulmonary, *etc.* Hematopoietic cells, which include any of the nucleated cells which may be involved with the lymphoid or myelomonocytic lineages and members of the T- and B-cell lineages, macrophages and monocytes, myoblasts and fibroblasts are of particular interest. Also of interest are stem and progenitor cells, such as hematopoietic, neural, stromal, muscle, hepatic, pulmonary and gastrointestinal progenitor cells.

The cells can be autologous cells, syngeneic cells, allogenic cells and even in some cases, xenogeneic cells. The cells may be modified by changing the major histocompatibility complex ("MHC") profile, by inactivating  $\beta_2$ -microglobulin to prevent the formation of functional Class I MHC molecules, by inactivating Class II molecules, by providing for expression of one or more MHC molecules, by enhancing or inactivating cytotoxic capabilities, by enhancing or inhibiting the expression of genes associated with the cytotoxic activity, or the like.

In some instances specific clones or oligoclonal cells may be of interest, where the cells have a particular specificity, such as T cells and B cells having a specific antigen specificity or homing target site specificity.

## V. Ligands

A wide variety of ligands, including both naturally occurring and synthetic substances, can be used in this invention to effect multimerization of the chimeric protein molecules. Applicable and readily observable or measurable criteria for selecting a ligand are: (A) the ligand is physiologically acceptable (i.e., lacks undue toxicity towards the cell or animal for which it is to be used), (B) it has a reasonable therapeutic dosage range, (C) desirably (for applications in whole animals, including gene therapy applications), it can be

taken orally (is stable in the gastrointestinal system and absorbed into the vascular system), (D) it can cross the cellular and other membranes, as necessary, and (E) binds to the receptor domain with reasonable affinity for the desired application. A first desirable criterion is that the compound is relatively  
5 physiologically inert, but for its activating capability with the receptors. The less the ligand binds to native receptors and the lower the proportion of total ligand which binds to native receptors, the better the response will normally be. Particularly, the ligand should not have a strong biological effect on native proteins. For the most part, the ligands will be non-peptide and non-nucleic  
10 acid.

The subject compounds will for the most part have two or more units, where the units can be the same or different, joined together through a central linking group. The "units" will be individual moieties (e.g., FK506, FK520, cyclosporin A, a steroid, etc.) capable of binding the receptor domain. Each of  
15 the units will usually be joined to the linking group through the same reactive moieties, at least in homodimers or higher order homo-multimers.

As indicated above, there are a variety of naturally-occurring receptors for small non-proteinaceous organic molecules, which small organic molecules fulfill the above criteria, and can be dimerized at various sites to provide a  
20 ligand according to the subject invention. Substantial modifications of these compounds are permitted, so long as the binding capability is retained and with the desired specificity. Many of the compounds will be macrocyclics, e.g. macrolides. Suitable binding affinities will be reflected in  $K_d$  values well below  $10^{-4}$ , preferably below  $10^{-6}$ , more preferably below about  $10^{-7}$ , although binding  
25 affinities below  $10^{-9}$  or  $10^{-10}$  are possible, and in some cases will be most desirable.

Currently preferred ligands comprise multimers, usually dimers, of compounds capable of binding to an FKBP protein and/or to a cyclophilin protein. Such ligands includes homo- and heteromultimers (usually 2-4, more  
30 usually 2-3 units) of cyclosporin A, FK506, FK520, and rapamycin, and derivatives thereof, which retain their binding capability to the natural or mutagenized binding domain. Many derivatives of such compounds are already

known, including synthetic high affinity FKBP ligands, which can be used in the practice of this invention. See Holt et al., *J. Am. Chem. Soc.* 1993, 115, 9925-9935. Sites of interest for linking of FK506 and analogs thereof include positions involving annular carbon atoms from about 17 to 24 and substituent  
5 positions bound to those annular atoms, e.g. 21 (allyl), 22, 37, 38, 39 and 40, or 32 (cyclohexyl), while the same positions except for 21 are of interest for FK520. For cyclosporin, sites of interest include MeBmt, position 3 and position 8.

Of particular interest are modifications to the ligand which change its  
10 binding characteristics, particularly with respect to the ligand's naturally occurring receptor. Concomitantly, one would change the binding protein to accommodate the change in the ligand. For example, one can modify the groups at position 9 or 10 of FK506 (see Van Duyne et al. (1991) *Science* 252, 839), so as to increase their steric requirement, by replacing the hydroxyl with  
15 a group having greater steric requirements, or by modifying the carbonyl at position 10, replacing the carbonyl with a group having greater steric requirements or functionalizing the carbonyl, e.g. forming an N-substituted Schiff's base or imine, to enhance the bulk at that position. Various functionalities which can be conveniently introduced at those sites are alkyl  
20 groups to form ethers, acylamido groups, N-alkylated amines, where a 2-hydroxyethylimine can also form a 1,3-oxazoline, or the like. Generally, the substituents will be from about 1 to 6, usually 1 to 4, and more usually 1 to 3 carbon atoms, with from 1 to 3, usually 1 to 2 heteroatoms, which will usually be oxygen, sulfur, nitrogen, or the like. By using different derivatives of the  
25 basic structure, one can create different ligands with different conformational requirements for binding. By mutagenizing receptors, one can have different receptors of substantially the same sequence having different affinities for modified ligands not differing significantly in structure.

Other ligands which can be used are steroids. The steroids can be  
30 multimerized, so that their natural biological activity is substantially diminished without loss of their binding capability with respect to a chimeric protein containing one or more steroid receptor domains. By way of non-limiting

example, glucocorticoids and estrogens can be so used. Various drugs can also be used, where the drug is known to bind to a particular receptor with high affinity. This is particularly so where the binding domain of the receptor is known, thus permitting the use in chimeric proteins of this invention of *only* the binding domain, rather than the entire native receptor protein. For this purpose, enzymes and enzyme inhibitors can be used.

#### A. Linkers

Various functionalities can be involved in the linking, such as amide groups, including carbonic acid derivatives, ethers, esters, including organic and inorganic esters, amino, or the like. To provide for linking, the particular monomer can be modified by oxidation, hydroxylation, substitution, reduction, *etc.*, to provide a site for coupling. Depending on the monomer, various sites can be selected as the site of coupling.

The multimeric ligands can be synthesized by any convenient means, where the linking group will be at a site which does not interfere with the binding of the binding site of a ligand to the receptor. Where the active site for physiological activity and binding site of a ligand to the receptor domain are different, it will usually be desirable to link at the active site to inactivate the ligand. Various linking groups can be employed, usually of from 1-30, more usually from about 1-20 atoms in the chain between the two molecules (other than hydrogen), where the linking groups will be primarily composed of carbon, hydrogen, nitrogen, oxygen, sulphur and phosphorous. The linking groups can involve a wide variety of functionalities, such as amides and esters, both organic and inorganic, amines, ethers, thioethers, disulfides, quaternary ammonium salts, hydrazines, *etc.* The chain can include aliphatic, alicyclic, aromatic or heterocyclic groups. The chain will be selected based on ease of synthesis and the stability of the multimeric ligand. Thus, if one wishes to maintain long-term activity, a relatively inert chain will be used, so that the multimeric ligand link will not be cleaved. Alternatively, if one wishes only a short half-life in the blood stream, then various groups can be employed which are readily cleaved, such as esters and amides, particularly peptides, where

circulating and/or intracellular proteases can cleave the linking group.

Various groups can be employed as the linking group between ligands, such as alkylene, usually of from 2 to 20 carbon atoms, azalkylene (where the nitrogen will usually be between two carbon atoms), usually of from 4 to 18 carbon atoms), N-alkylene azalkylene (see above), usually of from 6 to 24 carbon atoms, arylene, usually of from 6 to 18 carbon atoms, ardiakylene, usually of from 8 to 24 carbon atoms, bis-carboxamido alkylene of from about 8 to 36 carbon atoms, *etc.* Illustrative groups include decylene, octadecylene, 3-azapentylene, 5-azadecylene, N-butylene 5-azanonylene, phenylene, xylylene, p-dipropylenebenzene, bis-benzoyl 1,8-diaminooctane and the like. Multivalent or other ligand molecules containing linker moieties as described above can be evaluated with chimeric proteins of this invention bearing corresponding receptor domains using materials and methods described in the examples which follow.

#### 15 B. Ligand Characteristics

For intracellular binding domains, the ligand will be selected to be able to be transferred across the membrane in a bioactive form, that is, it will be membrane permeable. Various ligands are hydrophobic or can be made so by appropriate modification with lipophilic groups. Particularly, the linking  
20 bridge can serve to enhance the lipophilicity of the ligand by providing aliphatic side chains of from about 12 to 24 carbon atoms. Alternatively, one or more groups can be provided which will enhance transport across the membrane, desirably without endosome formation.

In some instances, multimeric ligands need not be employed. For  
25 example, molecules can be employed where two different binding sites provide for dimerization of the receptor. In other instances, binding of the ligand can result in a conformational change of the receptor domain, resulting in activation, *e.g.* multimerization, of the receptor. Other mechanisms may also be operative for inducing the signal, such as binding a single receptor with a  
30 change in conformation resulting in activation of the cytoplasmic domain.



### C. Ligand Antagonists

Monomeric ligands can be used for reversing the effect of the multimeric ligand, i.e., for preventing, inhibiting or disrupting multimer formation or maintenance. Thus, if one wishes to rapidly terminate the effect of cellular activation, a monomeric ligand can be used. Conveniently, the parent ligand moiety can be modified at the same site as the multimer, using the same procedure, except substituting a monofunctional compound for the polyfunctional compound. Instead of the polyamines, monoamines, particularly of from 2 to 20 (although they can be longer), and usually 2 to 12, carbon atoms can be used, such as ethylamine, hexylamine, benzylamine, *etc.* Alternatively, the monovalent parent compound can be used, in cases, or at dosage levels, in which the parent compound does not have undue undesirable physiological activity such as immunosuppression, mitogenesis, toxicity, and so forth.

### D. Illustrative hetero-multimerizing (HED) and homo-multimerizing (HOD) reagents with "bumps" that can bind to mutant receptors containing compensatory mutations

As discussed above, one can prepare modified HED/HOD reagents that will fail to bind appreciably to their wild type receptors (e.g., FKBP12) due to the presence of substituents ("bumps") on the reagents that sterically clash with sidechain residues in the receptor's binding pocket. One may also make corresponding receptors that contain mutations at the interfering residues ("compensatory mutations") and therefore gain the ability to bind ligands with bumps. Using "bumped" ligand moieties and receptor domains bearing compensatory mutations should enhance the specificity and thus the potency of these reagents. Bumped reagents should not bind to the endogenous, wild type receptors, which can otherwise act as a "buffer" toward dimerizers based on natural ligand moieties. In addition, the generation of novel receptor-ligand pairs should simultaneously yield the HED reagents that will be used when heterodimerization is required. example, regulated vesicle fusion may be achieved by inducing the heterodimerization of syntaxin (a plasma membrane

fusion protein) and synaptobrevin (a vesicle membrane fusion protein) using a HED reagent. This would not only provide a research tool, but could also serve as the basis of a gene therapy treatment for diabetes, using appropriately modified secretory cells.

5 As an illustration of "Bumped FK1012s" C10 acetamide and formamide derivatives of FK506 were prepared. See Figure 16 and Spencer et al., "Controlling Signal Transduction with Synthetic Ligands," *Science* 262 (1993) 1019-1024 for additional details concerning the syntheses of FK1012s A-C and FK506M. Two classes of bumped FK1012s were also created: one with a bump  
10 at C10 and another at C9. The R- and S-isomers of the C10 acetamide and formamide derivatives have been synthesized according to the reaction sequence shown in Figure 16B. These bumped derivatives have lost at least three orders of magnitude in their binding affinity towards FKBP12 (Figure 16B). The affinities were determined by measuring the ability of the derivatives to inhibit  
15 FKBP12's rotamase activity.

A representative member of a second class of C9-bumped derivatives is the spiro-epoxide (depicted in Figure 16C), which has been prepared by adaptation of known procedures. See e.g. Fisher et al., *J Org Chem* 56 8(1991): 2900-7 and Edmunds et al., *Tet. Lett.* 32 48 (1991):819-820. A particularly interesting series  
20 of C9 derivatives are characterized by their  $sp^3$  hybridization and reduced oxidation state at C9. Several such compounds have been synthesized according to the reactions shown in Figure 16C.

It should be appreciated that heterodimers (and other heteromultimerizers) must be constructed differently than the homodimers, at least  
25 for applications where homodimer contamination could adversely affect their successful use. One illustrative synthetic strategy developed to overcome this problem is outlined in Figure 16D. Coupling of mono alloc-protected 1,6-hexanediamine (Stahl et al., *J Org Chem* 43 11 (1978): 2285-6) with a derivatized form of FK506 in methylene chloride with an excess of triethylamine gave an  
30 alloc-amine-substituted FK506 in 44% yield. This intermediate can now be used in the coupling with any activated FK506 (or bumped-FK506) molecule. Deprotection with catalytic tetrakis-triphenylphosphine palladium in the

presence of dimedone at room temperature in THF removes the amine protecting group. Immediate treatment with an activated FK506 derivative, followed by desilylation leads to a dimeric product. This technique has been used to synthesize the illustrated HOD and HED reagents.

5    **E. Illustrative Cyclosporin-based reagents**

Cyclosporin A (CsA) is a cyclic undecapeptide that binds with high affinity (6nM) to its intracellular receptor cyclophilin, an 18 kDa monomeric protein. The resulting complex, like the FKBP12-FK506 complex, binds to and inactivates the protein phosphatase calcineurin resulting in the  
10 immunosuppressive properties of the drug. As a further illustration of this invention, CsA has been dimerized *via* its MeBmt1 sidechain in 6 steps and 35% overall yield to give (CsA)<sub>2</sub> (Figure 17, steps 1-4 were conducted as reported in Eberle et al., *J Org Chem* 57 9 (1992): 2689-91). As with FK1012s, the site for dimerization was chosen such that the resulting dimer can bind to  
15 two molecules of cyclophilin yet cannot bind to calcineurin following cyclophilin-binding. (CsA)<sub>2</sub> was found to bind to cyclophilin A with 1:2 stoichiometry. Hence, (CsA)<sub>2</sub>, like FK1012s, does not inhibit signaling pathways and is thus neither immunosuppressive nor toxic at concentrations useful for practicing this invention.

20    **VI. Genes for Regulatable Blocking of Gene Expression or Function and for Gene Elimination**

**A. Transcription Initiation Region for the Blocking Gene Constructs**

Blocking gene constructs encode gene products which are capable of blocking expression of target genes, the interfering with the function of the  
25 gene products encoded by target gene or eliminating the gene from the host cell entirely. Blocking genes of this latter type may genes which encode gene products such as the Cre recombinase, whose expression leads to elimination of a target gene appropriately flanked by loxP sequences in host cells. Constructs encoding the blocking gene products of the present invention have a responsive  
30 element in the 5' region, which responds to ligand-mediated multimerization of

the chimeric receptor protein, presumably via the generation and transduction of a transcription initiation signal as discussed *infra*. Therefore, it will be necessary to select at least one transcription initiation system, e.g. transcription factor, which is activated either directly or indirectly, by the cytoplasmic domain or can be activated by association of two domains. It will also be necessary to select at least one promoter region which is responsive to the resulting transcription initiation system. Either the promoter region or the gene under its transcriptional control need be selected. In other words, an action domain can be selected for the chimeric proteins, which is encoded by a "first" series construct, based on the role of that action domain in initiating transcription via a given promoter or responsive element. See e.g. Section III(A) "Cytoplasmic domains", above.

Where the responsive element is known, it can be included in the blocking gene construct to provide an expression cassette for integration into the genome either as an episome or by chromosomal integration. It is not necessary to have isolated the particular sequence of the responsive element, so long as a gene is known, which is transcriptionally activated by the cytoplasmic domain upon natural ligand binding to the protein comprising the cytoplasmic domain. Homologous recombination is then used for insertion of the gene of interest downstream from the promoter region such that the inserted gene is under the transcriptional regulation of the endogenous promoter region.

Where the sequence of a specific responsive element is known, it can be used in conjunction with a different transcription initiation region, which has other advantageous or desired properties, such as a high or low rate of transcription, binding by particular transcription factors, including tissue specific binding factors, and the like.

The expression construct will therefore have at its 5' end in the direction of transcription, the responsive element and the promoter sequence which allows induced transcription initiation of a target gene of interest, usually a therapeutic gene. The transcriptional termination region is not as important, and can be used to enhance the lifetime of or make short half-lived mRNA by inserting AU sequences which serve to reduce the stability of the

mRNA and, as a consequence, limit the period of activity of the protein. Any region can be employed which provides for the necessary transcriptional termination, and as appropriate, translational termination.

5 The responsive element can be a single sequence or it can be a repeated sequence, but it would usually not be repeated more than about 5 times, often not more than about 3 times.

Homologous recombination can be used to remove or inactivate endogenous transcriptional control sequences, including promoter, enhancer and other responsive elements, which are responsive to the multimerization of  
10 Group (1) and Group (2) chimeric proteins as discussed above. Additionally, homologous recombination can be used to insert responsive transcriptional control sequences upstream of a desired endogenous gene.

#### B. Target Gene

A wide variety of genes can be employed as the target gene. The target  
15 gene can be any sequence of interest, the absence of which provides a desired phenotype. The target gene can encode a surface membrane protein, a secreted protein, a cytoplasmic protein, or there can be a plurality of target genes which can express different types of products. The encoded proteins can be involved in homing, cytotoxicity, proliferation, immune response, inflammatory  
20 response, clotting or dissolving of clots, hormonal regulation, or the like (See Table 1). The target gene may alternatively encode a product of unknown function, in which case the modified cells or organisms in which the target gene is regulatably obstructed can be used to identify or study that function. In addition, the target gene need not be endogenous to the engineered cell—in  
25 some embodiments it is a gene of an infective agent e.g. bacteria or viruses. In such embodiments, it is a viral or bacterial gene or gene product which is regulatably obstructed.

#### C. Blocking Gene

In the practice of this invention there are several options and  
30 considerable flexibility with regard to choice of blocking agent. In all cases the

gene encoding the blocking agent is expressed under the transcriptional regulation of an element responsive to the multimerization of chimeric proteins, as described in detail elsewhere. The blocking gene may encode an anti-sense message or a ribozyme, an antibody or related form thereof, or a dominant negative form of the target gene product. Additionally, the blocking gene can be a gene that is capable of eliminating the target gene from the host genome completely, such as the gene encoding the protein Cre, which produces Cre recombinase. Expression of this recombinase leads to elimination of a target gene appropriately flanked by "loxP" sequences in the host cells.

**(i) Antisense messages and ribozymes for blocking target gene expression**

When the target gene sequence is known, its expression can be blocked by ligand-regulated expression of an antisense message or ribozyme. An antisense message or a ribozyme contains sufficient sequence complementary to the target gene such that it specifically recognizes the target message and blocks its expression. For a recent review containing useful background information and guidance, see Altman, "RNA enzyme-directed gene therapy," *Proc. Natl. Acad. Sci. USA* 90 (1993) 10898-10900 and papers cited therein, including Yu et al., "A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1," *Proc. Natl. Acad. Sci. USA* 90 (1993) 6340-6344. See also Efrat et al., Ribozyme-mediated attenuation of pancreatic  $\beta$ -cell glucokinase expression in transgenic mice results in impaired glucose-induced insulin secretion., *Proc. Natl. Acad. Sci. USA* 91, 2051-2055.

**(ii) Intracellular Expression of Antibodies to Block Gene Function**

The function of a target gene product can be blocked by ligand-regulated expression of an antibody, antibody fragment or other antibody like moiety, that specifically recognizes the encoded target protein and blocks its cellular function. This is done by expressing a gene encoding a neutralizing or otherwise blocking antibody against the target gene product. By regulatably expressing such an antibody gene, one regulatably obstructs the functioning of the target protein. As in other embodiments, this may be effected in a cell-type

specific manner by expressing the chimeric proteins using a cell-type specific promoter.

In this way, only cells that express the multimerizable chimeric proteins are capable of expressing the antibody, and do so only in the presence of or  
5 following exposure to the multimerization agent.

Examples of intracellularly expressed antibody moieties blocking a gene product function include an antibody to HIV I gp120 protein expressed in a mammalian cell (Marasco et al. PNAS 90:7889 1993) and an anti-p21ras antibody expressed in *Xenopus* oocytes (Biocca et al. BBRC 197: 422 1993).  
10 Intracellular expression of the anti-gp120 antibody blocked processing of the envelop precursor and reduced the infectivity of HIV-1 particles. The anti-ras antibody blocked insulin-mediated meiotic maturation of *Xenopus* oocytes.

The antibody should be selected such that it binds to the target gene or gene product and blocks its cellular function. A preferred form of the antibody  
15 is the so-called *single chain* form, since this ensures that the heavy and light chains will associate within the cell. Other configurations, such as two chain antibodies, particularly two chain  $F_{ab}$ s, or  $F_v$  fragments, or antibody chains fused to other proteins are also possible. The antibody may need to be targeted to the same compartment as the desired gene product to be disrupted. This can  
20 be accomplished by fusing a localization sequence to the antibody coding sequence. This could be at the N-terminus, C-terminus, or embedded within the antibody moiety amino acid sequence.

The technology of generating recombinant antibodies, including single-chain antibodies is well known in the art. For a recent review, see Huston et al., *Int Rev. Immunol.* 10 (1993) 195.  
25

### (iii) Interference by A Dominant Negative Gene Product

Protein-protein interactions that are critical for a cellular process can be selectively blocked by expression of a non-functional variant of one of the protein partners. For example, raf-1 is a serine/threonine protein kinase that  
30 functions in growth factor-stimulated proliferation pathway (Schaap et al. J. Biol. Chem. 268: 20232 1993). It is composed to two domains, an N-terminal

regulatory domain and C-terminal kinase domain. Constitutive overexpression of the N-terminal domain of p74raf-1 in cultured cells blocked mitogenesis induced by growth factors. This domain also interfered with an oncogenic variant of p21ras. Such a system could be useful for models of cancer or the  
5 role of growth factors on cellular proliferation.

Other examples of dominant negative gene products include certain variants of steroid receptors, growth factor receptors having an inactive protein kinase or lacking the protein kinase domain altogether, cell surface receptors having a non-functional extracellular ligand binding domain or intracellular  
10 cytoplasmic domain, transcription factor variants that lack a DNA binding domain and/or a transactivation domain.

Dominant negative proteins typically disrupt the normal function of a target protein by sequestering it away from its normal partner. Dominant negative proteins can be constructed by random mutagenesis, by selective  
15 deletion of gene segments, or by a rational protein engineering where the domain structure and function of the protein is understood. Often the dominant negative protein is an inactive version of a protein with enzymatic activity.

One important requirement is that the dominant negative protein be  
20 overexpressed relative to its normal counterpart. The increased expression afforded by the ligand-regulated transcriptional activation of our invention makes this a particularly useful application of the technology.

#### iv. Intracellular Expression of Cre to Eliminate a Target Gene

The Cre constructs and floxed target gene constructs for use in this  
25 embodiment of the invention will generally be as described by Barinaga (1994) and by Gu et al. (1994), cited above, and references cited therein, with the proviso that the Cre-encoding DNA sequence will be linked to a promoter/enhancer sequence responsive to multimerization of Group (1) chimeras or to a DNA sequence to which multimerized Group (2) chimeras are  
30 capable of binding and initiating Cre transcription.

The modified cells or organisms in which the target gene is regulatably



eliminated can be used to identify or study the function of the gene that is eliminated either at the cellular level or at the level of the organism.

Cre constructs will have a responsive element in the 5' region, which responds to ligand-mediated multimerization of the chimeric receptor protein, presumably via the generation and transduction of a transcription initiation signal as discussed *infra*. Therefore, it will be necessary to select at least one transcription initiation system, which utilizes at least one transcription factor, which is activated either directly or indirectly, by the cytoplasmic domain or can be activated by association of two domains. It will also be necessary to select at least one promoter region which is responsive to the resulting transcription initiation system. Either the promoter region or the gene under its transcriptional control need be selected. In other words, an action domain can be selected for the chimeric proteins (encoded by a "first" series construct) based on the role of that action domain in initiating transcription via a given promoter or responsive element. See e.g. Section III(A) "Cytoplasmic domains", above.

Where the responsive element is known, it can be included in the Cre gene construct to provide an expression cassette for integration into the genome whether as an episome or by incorporation into the chromosome. It is not necessary to have isolated the particular sequence of the responsive element, so long as a gene is known which is transcriptionally activated by the cytoplasmic domain upon natural ligand binding to the protein comprising the cytoplasmic domain. Homologous recombination could then be used for insertion of the gene of interest downstream from the promoter region to be under the transcriptional regulation of the endogenous promoter region. Where the specific responsive element sequence is known, that can be used in conjunction with a different transcription initiation region, which can have other aspects, such as a high or low activity as to the rate of transcription, binding of particular transcription factors and the like.

The expression construct will therefore have at its 5' end in the direction of transcription, the responsive element and the promoter sequence which allows for induced transcription initiation of a target gene of interest,

usually a therapeutic gene. The transcriptional termination region is not as important, and can be used to enhance the lifetime of or make short half-lived mRNA by inserting AU sequences which serve to reduce the stability of the mRNA and, therefore, limit the period of action of the protein. Any region  
5 can be employed which provides for the necessary transcriptional termination, and as appropriate, translational termination.

The responsive element can be a single sequence or can be multimerized, usually having not more than about 5 repeats, usually having about 3 repeats.

10 Homologous recombination can also be used to remove or inactivate endogenous transcriptional control sequences, including promoter and/or responsive elements, which are responsive to the multimerization event, and/or to insert such responsive transcriptional control sequences upstream of a desired endogenous gene.

15 A wide variety of genes can be employed as the target gene to be eliminated from selected host cells. The target gene can be any sequence of interest, the absence of which provides a desired phenotype. The target gene can encode a surface membrane protein, a secreted protein, a cytoplasmic protein, or there can be a plurality of target genes which can express different  
20 types of products. The encoded proteins can be involved in homing, cytotoxicity, proliferation, immune response, inflammatory response, clotting or dissolving of clots, hormonal regulation, or the like (See Table 1). The target gene may alternatively encode a product of unknown function, in which case the modified cells or organisms in which the target gene is regulatably  
25 eliminated can be used to identify or study that function.

#### v. Regulated Apoptosis

In many situations it may be desirable to kill the genetically modified recombinant cells, such as where one wishes to terminate the treatment provided by the modified cells, where the cells have become neoplastic,  
30 particularly in a patient, where a genetic therapy has been deleterious rather than beneficial or where the removal of the cells from a subject, particularly a recombinant animal, after the engineered cells have expressed a desired protein,

may be of interest for research. For this purpose one may provide for the expression of the Fas antigen or TNF receptor fused to a binding domain. (See Watanabe-Fukunaga et al., *Nature* 356 (1992) 314-317.) Cells containing such constructs are readily eliminated through apoptosis following exposure of the  
5 cells to a ligand capable of oligomerizing the primary chimeras. Constructs encoding the primary chimera may be designed for constitutive expression using conventional materials and methods, so that the modified cells have such proteins on their surface or present in their cytoplasm. Alternatively, one can provide for controlled expression, where the same or different oligomerizing  
10 ligand can initiate expression of the primary chimera and initiate apoptosis. By providing for the cytoplasmic portions of the Fas antigen or TNF receptor in the cytoplasm joined to binding regions different from the binding regions associated with expression of a target gene of interest, one can kill the modified cells under controlled conditions.

## 15 VII. Introduction of Constructs into Cells

The constructs described herein can be introduced as one or more DNA molecules or constructs, where there will usually be at least one marker and there may be two or more markers, which will allow for selection of host cells which contain the constructs. The constructs can be prepared in conventional  
20 ways, where the genes and regulatory regions may be isolated, as appropriate, ligated, cloned in an appropriate cloning host, analyzed by restriction or sequencing, or other convenient means. Particularly, using PCR, individual fragments including all or portions of a functional unit may be isolated, where one or more mutations may be introduced using "primer repair", ligation, *in*  
25 *vitro* mutagenesis, *etc.* as appropriate. Once the constructs are completed and have been demonstrated to contain the appropriate, desired sequences, they may be introduced into the host cell by any convenient means. The constructs may be integrated and packaged into non-replicating, defective viral genomes like adenovirus, adeno-associated virus (AAV), or herpes simplex virus (HSV) or  
30 others, including retroviral vectors, for infection or transduction into cells. The constructs may include viral sequences for transfection, if desired.

Alternatively, the construct may be introduced by fusion, electroporation, biolistics, transfection, lipofection, or the like. The host cells will usually be grown and expanded in culture before introduction of the construct(s), followed by the appropriate treatment for introduction of the construct(s) and  
5 integration of the construct(s). The cells will then be expanded and screened by virtue of a marker present in the construct. Various markers which may be used successfully include *hprt*, neomycin resistance, thymidine kinase, hygromycin resistance, etc.

In some instances, one may have a target site for homologous  
10 recombination, where it is desired that a construct be integrated at a particular locus. For example, an endogenous gene, at the same locus or elsewhere, can be deleted and/or replaced with a recombinant construct of this invention, using materials and methods known in the art for homologous recombination. The recombinant constructs of this invention also can be used to introduce the  
15 floxed target gene into particular cells, using materials and methods known in this art. For homologous recombination, one may generally use either  $\Omega$  or O-vectors. See, for example, Thomas and Capecchi, *Cell* (1987) 51, 503-512; Mansour, *et al.*, *Nature* (1988) 336, 348-352; and Joyner, *et al.*, *Nature* (1989) 338, 153-156. Gu *et al.* (1994) provide additional methods which can be used  
20 for introduction of the floxed target gene.

The constructs may be introduced as a single DNA molecule encoding all of the genes, or as different DNA molecules having one or more genes. The constructs may be introduced simultaneously or consecutively, each with the same or different markers. In an illustrative example, one construct would  
25 contain a therapeutic gene under the control of a specific responsive element (e.g. NFAT), another encoding the receptor fusion protein comprising the signaling region fused to the ligand receptor domain (e.g. as in MZF3E). A third DNA molecule encoding a homing receptor or other product that increases the efficiency of delivery of the therapeutic product may also be  
30 introduced.

Vectors containing useful elements such as bacterial or yeast origins of replication, selectable and/or amplifiable markers, promoter/enhancer elements

for expression in prokaryotes or eukaryotes, etc. which may be used to prepare stocks of construct DNAs and for carrying out transfections are well known in the art, and many are commercially available.

### VIII. Administration of Cells and Ligands

5       The cells which have been modified with the DNA constructs may be grown in culture under selective conditions and cells which are selected as having the construct may then be expanded and further analyzed. For example, the polymerase chain reaction may be used for verifying the presence of the construct in the host cells. Once the modified host cells have been  
10 identified, they may then be grown in culture or introduced into a host organism, as appropriate for the purpose for which they were developed.

      Depending upon the nature of the particular modified cells, they may be introduced into a host organism, such as a mammal, in a wide variety of ways. Hematopoietic cells may be administered by injection into the vascular  
15 system, there being usually at least about  $10^4$  cells and generally not more than about  $10^{10}$ , more usually not more than about  $10^8$  cells. The number of cells which are administered will depend upon a number of circumstances, including the purpose of introducing the modified cells, the lifetime of the cells and the administration protocol used. For example, the number of administrations, the  
20 ability of the cells to multiply, the stability of the therapeutic agent, the physiologic need for the therapeutic agent, and the like will all be considered in determining the number of modified cells to be administered. With skin cells which may be used as a graft, the number of modified cells used would depend upon the size of the layer to be applied to the burn or other lesion. Generally,  
25 for myoblasts or fibroblasts, the number of cells will be at least about  $10^4$  and not more than about  $10^8$  and may be applied as a dispersion, generally being injected at or near the site of interest. The cells will usually be in a physiologically-acceptable medium.

      Instead of *ex vivo* modification of the cells, in many situations one may  
30 wish to modify cells *in vivo*. For this purpose, various techniques have been developed for modification of target tissue and cells *in vivo*. A number of virus

vectors have been developed, such as adenovirus and retroviruses, which allow for transfection and random integration of the virus into the host. See, for example, Dubensky et al. (1984) Proc. Natl. Acad. Sci. USA 81, 7529-7533; Kaneda et al., (1989) Science 243,375-378; Hiebert et al. (1989) Proc. Natl. Acad. Sci. USA 86, 3594-3598; Hatzoglu et al. (1990) J. Biol. Chem. 265, 17285-17293 and Ferry, et al. (1991) Proc. Natl. Acad. Sci. USA 88, 8377-8381. The vector may be administered by injection, e.g. intravascularly or intramuscularly, inhalation, or other parenteral mode.

In accordance with *in vivo* genetic modification, the manner of the modification will depend on the nature of the tissue, the efficiency of cellular modification required, the number of opportunities to modify the particular cells, the accessibility of the tissue to the DNA composition to be introduced, and the like. By employing an attenuated or modified retrovirus carrying a target transcriptional initiation region, one can activate the virus using one of the subject transcription factor constructs, so that the virus will be produced and transfect adjacent cells.

The DNA introduction need not result in integration in every case. In some situations, transient maintenance of the DNA introduced may be sufficient. In this way, one could have a short term effect, where cells could be introduced into the host and then turned on after a predetermined time, for example, after the cells have been able to home to a particular site.

The ligand providing for activation of the cytoplasmic domain may then be administered as desired. Depending upon the binding affinity of the ligand, the response desired, the manner of administration, the half-life, the number of cells present, various protocols may be employed. The ligand may be administered parenterally or orally. The number of administrations will depend upon the factors described above. The ligand may be taken orally as a pill, powder, or dispersion; buccally; sublingually; injected intravascularly, intraperitoneally, subcutaneously; by inhalation, or the like. The ligand (and monomeric compound) may be formulated using conventional methods and materials well known in the art for the various routes of administration. The precise dose and particular method of administration will depend upon the

above factors and be determined by the attending physician or human or animal healthcare provider. For the most part, the manner of administration will be determined empirically.

5 In the event that the activation by the ligand is to be reversed, the monomeric compound may be administered or other single binding site compound which can compete with the ligand. Thus, in the case of an adverse reaction or the desire to terminate the therapeutic effect, the monomeric binding compound can be administered in any convenient way, particularly intravascularly, if a rapid reversal is desired. Alternatively, one may provide for  
10 the presence of an inactivation domain (or transcriptional silencer) with a DNA binding domain. In another approach, cells may be eliminated through apoptosis via signalling through Fas or TNF receptor as discussed Example 4(B) below.

The particular dosage of the ligand for any application may be  
15 determined in accordance with the procedures used for therapeutic dosage monitoring, where maintenance of a particular level of expression is desired over an extended period of times, for example, greater than about two weeks, or where there is repetitive therapy, with individual or repeated doses of ligand over short periods of time, with extended intervals, for example, two weeks or  
20 more. A dose of the ligand within a predetermined range would be given and monitored for response, so as to obtain a time-expression level relationship, as well as observing therapeutic response. Depending on the levels observed during the time period and the therapeutic response, one could provide a larger or smaller dose the next time, following the response. This process would be  
25 iteratively repeated until one obtained a dosage within the therapeutic range. Where the ligand is chronically administered, once the maintenance dosage of the ligand is determined, one could then do assays at extended intervals to be assured that the cellular system is providing the appropriate response and level of the expression product.

30 It should be appreciated that the system is subject to many variables, such as the cellular response to the ligand, the efficiency of expression and, as appropriate, the level of secretion, the activity of the expression product, the

particular need of the patient, which may vary with time and circumstances, the rate of loss of the cellular activity as a result of loss of cells or expression activity of individual cells, and the like. Therefore, it is expected that individual patient would be monitored for the proper dosage for the individual, even if there were universal cells which could be administered to the population at large.

The subject methodology and compositions may be used for the study and/or treatment of a wide variety of conditions and indications. For example, B- and T-cells may be used in the investigation and/or treatment of cancer, infectious diseases, metabolic deficiencies, cardiovascular disease, hereditary coagulation deficiencies, autoimmune diseases, joint degenerative diseases, such as arthritis, pulmonary disease, kidney disease, endocrine abnormalities, *etc.* Various cells involved with structure, such as fibroblasts and myoblasts, may be used in the treatment and/or investigation of genetic deficiencies, such as connective tissue deficiencies, arthritis, hepatic disease, *etc.*

The following examples contain important additional information, exemplification and guidance which can be adapted to the practice of this invention in its various embodiments and the equivalents thereof. The examples are offered by way illustration and not by way limitation.

## Examples

### Cellular Transformations and Evaluation

#### Example 1: Induction of Isolated IL-2 Enhancer-Binding Transcription Factors by Cross-Linking the CD3 Chain of the T-Cell Receptor.

The plasmid pSXNeo/IL2 (IL2-SX) (Figure 1), which contains the placental secreted alkaline phosphatase gene under the control of human IL-2 promoter (-325 to +47; MCB(86) 6, 3042), and related plasmid variants (*i.e.* NFAT-SX, NFB-SX, OAP/Oct1-SX, and AP-1-SX) in which the reporter gene is under the transcriptional control of the minimal IL-2 promoter (-325 to -294 and -72 to +47) combined with synthetic oligomers containing various promoter elements (*i.e.* NFAT, NKB, OAP/Oct-1, and AP1, respectively), were made by three piece ligations of 1) pPL/SEAP (Berger, *et al.*, *Gene* (1988) 66,1) cut with SspI



and HindIII; 2) pSV2/Neo (Southern and Berg, *J. Mol. Appl. Genet.* (1982) 1, 332) cut with NdeI, blunted with Klenow, then cut with PvuI; and 3) various promoter-containing plasmids (i.e. NFAT-CD8, B-CD8,  $\alpha$ 12lacZ-Oct-1, AP1-LUCIF3H, or  $\alpha$ 15IL2) (described below) cut with PvuI and HindIII. NFAT-CD8 contains 3 copies of the NFAT-binding site (-286 to -257; *Genes and Dev.* (1990) 4, 1823) and  $\alpha$ 12lacZ-Oct contains 4 copies of the OAP/Oct-1/(ARRE-1) binding site (MCB, (1988) 8, 1715) from the human IL-2 enhancer; B-CD8 contains 3 copies of the NFB binding site from the murine light chain (EMBO (1990) 9, 4425) and AP1-LUCIF3H contains 5 copies of the AP-1 site (5'-TGA-CTCAGCGC-3') from the metallothionin promoter.

In each transfection, 5  $\mu$ g of expression vector, pCDL-SR (MCB 8, 466-72) (Tac-IL2 receptor -chain), encoding the chimeric receptor TAC/TAC/Z (TTZ) (PNAS 88, 8905-8909), was co-transfected along with various secreted alkaline phosphatase-based reporter plasmids (see map of pSXNeo/IL2 in Figure 1) in TAg Jurkat cells (a derivative of the human T-cell leukemia line Jurkat stably transfected with the SV40 large T antigen (Northrup, *et al.*, *J. Biol. Chem.* [1993])). Each reporter plasmid contains a multimerized oligonucleotide of the binding site for a distinct IL-2 enhancer-binding transcription factor within the context of the minimal IL-2 promoter or, alternatively, the intact IL-2 enhancer/promoter upstream of the reporter gene. After 24 hours, aliquots of cells (approximately  $10^5$ ) were placed in microtiter wells containing log dilutions of bound anti-TAC (CD25) mAb (33B3.1; AMAC, Westbrook, ME). As a positive control and to control for transfection efficiency, ionomycin (1  $\mu$ m) and PMA (25 ng/ml) were added to aliquots from each transfection. After an additional 14 hour incubation, the supernatants were assayed for the alkaline phosphatase activity and these activities were expressed relative to that of the positive control samples. The addition of 1 ng/ml FK506 dropped all activity due to NFAT to background levels, demonstrating that deactivations are in the same pathway as that blocked by FK506. Each data point obtained was the average of two samples and the experiment was performed several times with similar results. See Figure 5. The data show that with a known extracellular receptor, one obtains an appropriate response with a reporter gene

and different enhancers. Similar results were obtained when a MAb against the TcR complex (*i.e.* OKT3) was employed.

**Example 2: Inhibitory Activity of the Immunosuppressant Drugs FK506 and Cyclosporin A (CsA) or the Dimeric Derivative Compounds FK1012A (8), FK1012B (5), and CsA dimer (PB-1-218).**

Ionomycin ( $1\ \mu\text{M}$ ) and PMA ( $25\ \text{ng/ml}$ ) were added to  $10^5$  TAg-Jurkat cells. In addition, titrations of the various drugs were added. After 5 hours the cells were lysed in mild detergent (*i.e.* Triton X-100) and the extracts were incubated with the  $\beta$ -galactosidase substrate, MUG (methyl galactosidyl umbelliferone) for 1 hour. A glycine/EDTA stop buffer was added and the extracts assayed for fluorescence. Each data point obtained was the average of two samples and the experiment was performed several times with similar results. Curiously, FK1012B appears to augment mitogen activity slightly at the highest concentration (*i.e.*  $5\ \mu\text{g/ml}$ ); however, a control experiment shows that FK1012B is not stimulatory by itself. See Figure 6.

**Example 3. Activity of the Dimeric FK506 Derivative, FK1012A, on the Chimeric FKBP12/CD3 (1FK3) Receptor.**

$5\ \mu\text{g}$  of the eukaryotic expression vector, pBJ5, (based on pCDL-SR with a polylinker inserted between the 16S splice site and the poly A site), containing the chimeric receptor (1FK3), was co-transfected with  $4\ \mu\text{g}$  of the NFAT-inducible secreted alkaline phosphatase reporter plasmid, NFAT-SX. As a control,  $5\ \mu\text{g}$  of pBJ5 was used, instead of 1FK3/pBJ5, in a parallel transfection. After 24 hours, aliquots of each transfection containing approximately  $10^5$  cells were incubated with log dilutions of the drug, FK1012A, as indicated. As a positive control and to control for transfection efficiency, ionomycin ( $1\ \mu\text{M}$ ) and PMA ( $25\ \text{ng/ml}$ ) were added to aliquots from each transfection. After an additional 14 hour incubation, the supernatants were assayed for alkaline phosphatase activity and these activities were expressed relative to that of the positive control samples. The addition of  $2\ \text{ng/ml}$  FK506 dropped all stimulations to background levels, demonstrating that the activations are in the same pathway as that blocked by FK506. Hence,

FK506 or cyclosporin will serve as effective antidotes to the use of these compounds. Each data point obtained was the average of two samples and the experiment was performed several times with similar results. See Figure 7.

**Example 4A. Activity of the Dimeric FK506 Derivative, FK1012B, on the**  
5 **Myristoylated Chimeric CD3/FKBP12 (MZF3E) Receptor.**

A number of approaches to ligand design and syntheses have been successfully demonstrated, including positive results with FK506-based HOD reagents named "FK1012"s. FK1012s were found to achieve high affinity, 2:1 binding stoichiometry ( $K_d(1) = 0.1$  nM;  $K_d(2) = 0.8$  nM) and do not inhibit  
10 calcineurin-mediated TCR signaling. The ligands are neither "immunosuppressive" nor toxic (up to 0.1 mM in cell culture). Similarly, we have prepared a cyclosporin A-based homodimerizing agent, "(CsA)2" which binds to the CsA receptor, cyclophilin, with 1:2 stoichiometry, but which does not bind to calcineurin. Thus, like FK1012s, (CsA)2 does not inhibit signalling  
15 pathways and is thus neither immunosuppressive nor toxic.

These and other of the examples of ligand-mediated protein association resulted in the control of a signal transduction pathway. In an illustrative case, this was accomplished by creating an intracellular receptor comprised of a small fragment of Src sufficient for posttranslational myristoylation (M), the  
20 cytoplasmic tail of zeta ( $\zeta$ ; a component of the B cell receptor was also used), three consecutive FKBP12s (F3) and a flu epitope tag (E). Upon expressing the construct MZF3E (Figure 18) in human (Jurkat) T cells, it was confirmed that the encoded chimeric protein underwent FK1012-mediated oligomerization. The attendant aggregation of the zeta chains led to signaling via the endogenous  
25 TCR-signaling pathway (Figure 15), as evidenced by secretion of alkaline phosphatase (SEAP) in response to an FK1012 ( $EC_{50} = 50$  nM). The promoter of the SEAP reporter gene was constructed to be transcriptionally activated by nuclear factor of activated T cells (NFAT), which is assembled in the nucleus following TCR-signaling. FK1012-induced signaling can be terminated by a  
30 deaggregation process induced by a nontoxic, monomeric version of the ligand called FK506-M.

Specifically, 5  $\mu$ g of the eukaryotic expression vector, pBJ5, containing a myristoylated chimeric receptor was co-transfected with 4  $\mu$ g NFAT-SX. MZE, MZF1E, MZF2E and MZF3E contain 0, 1, 2, or 3 copies of FKBP12, respectively, downstream of a myristoylated CD3 cytoplasmic domain (see Figure 2). As a control, 5  $\mu$ g of pBJ5 was used in a parallel transfection. After 24 hours, aliquots of each transfection containing approximately  $10^5$  cells were incubated with log dilutions of the drug, FK1012B, as indicated. As a positive control and to control for transfection efficiency, ionomycin (1  $\mu$ m) and PMA (25 ng/ml) were added to aliquots from each transfection. After an additional 12 hour incubation, the supernatants were assayed for alkaline phosphatase activity and these activities were expressed relative to that of the positive control samples. The addition of 1 ng/ml FK506 dropped all stimulations to near background levels, demonstrating that the activations are in the same pathway as that blocked by FK506. This result is further evidence of the reversibility of the subject cell activation. Each data point obtained was the average of two samples and the experiment was performed several times with similar results. See Figure 8. The myristoylated derivatives respond to lower concentrations of the ligand by about an order of magnitude and activate NFAT dependent transcription to comparable levels, but it should be noted that the ligands are different. Compare Figs. 7 and 8.

**In vivo FK1012-induced protein dimerization** Confirmation was then obtained that intracellular aggregation of the MZF3E receptor is indeed induced by the FK1012. The influenza haemagglutinin epitope-tag (flu) of the MZF3E-construct was therefore exchanged with a different epitope-tag (flag-M2). The closely related chimeras, MZF3E<sub>flu</sub> and MZF3E<sub>flag</sub>, were co-expressed in Jurkat T cells. Immunoprecipitation experiments using anti-Flag-antibodies coupled to agarose beads were performed after the cells were treated with FK1012A. In the presence of FK1012A (1 $\mu$ M) the protein chimera MZF3E<sub>flag</sub> interacts with MZF3E<sub>flu</sub> and is co-immunoprecipitated with MZF3E<sub>flu</sub>. In absence of FK1012A, no co-immunoprecipitation of MZF3E<sub>flu</sub> is observed. Related experiments with FKBP monomer constructs MZF1E<sub>flu</sub> and MZF1E<sub>flag</sub>, which do not signal, revealed that they are also dimerized by FK1012A. This

reflects the requirement for aggregation observed with both the endogenous T cell receptor and our artificial receptor MZF3E.

FK1012-induced protein-tyrosine phosphorylation The intracellular domains of the TCR, CD3 and zeta-chains interact with cytoplasmic protein tyrosine kinases following antigen stimulation. Specific members of the Src family (lck and/or fyn) phosphorylate one or more tyrosine residues of activation motifs within these intracellular domains (tyrosine activation motif, TAM). The tyrosine kinase ZAP-70 is recruited (via its two SH2 domains) to the tyrosine phosphorylated T-cell-receptor, activated, and is likely to be involved in the further downstream activation of phospholipase C. Addition of either anti-CD3 mAb or FK1012A to Jurkat cells stably transfected with MZF3E resulted in the recruitment of kinase activity to the zeta-chain as measured by an in vitro kinase assay following immunoprecipitation of the endogenous T cell receptor zeta chain and the MZF3E-construct, respectively. Tyrosine phosphorylation after treatment of cells with either anti-CD3 mAb or FK1012 was detected using monoclonal alpha-phosphotyrosine antibodies. Whole cell lysates were analyzed at varying times after stimulation. A similar pattern of tyrosine-phosphorylated proteins was observed after stimulation with either anti-CD3 MAb or FK1012. The pattern consisted of a major band of 70 kDa, probably ZAP-70, and minor bands of 120 kDa, 62 kDa, 55 kDa and 42 kDa.

#### **Example 4(B): Regulation of Programmed Cell Death with Immunophilin-Fas Antigen Chimeras**

The Fas antigen is a member of the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily of cell surface receptors. Crosslinking of the Fas antigen with antibodies to its extracellular domain activates a poorly understood signaling pathway that results in programmed cell death or apoptosis. The Fas antigen and its associated apoptotic signaling pathway are present in most cells including possibly all tumor cells. The pathway leads to a rapid and unique cell death (2 h) that is characterized by condensed cytoplasm, the absence of an inflammatory response and

fragmentation of nucleosomal DNA, none of which are seen in necrotic cell death.

We have also developed a second, inducible signaling system that leads to apoptotic cell death. Like the MZF3E pathway, this one is initiated by  
5 activating an artificial receptor that is the product of a constitutively expressed "responder" gene. However, the new pathway differs from the first in that our HOD reagents induce the synthesis of products of an endogenous pathway rather than of the product of a transfected, inducible (e.g., reporter) gene.

Gaining control over the Fas pathway presents significant opportunities  
10 for biological research and medicine. Transgenic animals can be designed with "death" responder genes under the control of cell-specific promoters. Target cells may then be chemically ablated in the adult animal by administering a HOD reagent to the animal. In this way, the role of specific brain cells in memory or cognition or immune cells in the induction and maintenance of  
15 autoimmune disorders could be assessed. Death responder genes may also be introduced into tumors using the human gene therapy technique developed by M. Blaese and co-workers (Culver et al., *Science* 256 5063 (1992) 1550-2) and then subsequently activated by treating the patient with a HOD reagent in a manner similar to the "gancyclovir" gene therapy clinical trials recently  
20 reported for the treatment of brain tumors. Finally, we contemplate the co-administration of a death-responder gene together with the therapeutic gene in the practice of gene therapy. This would provide a "failsafe" component to gene therapy. If something were to go awry, for example, if an integration - induced loss of a tumor suppressor gene which could lead to cancer were to  
25 occur, the gene therapy patient could take a "failsafe" pill that would kill all transfected cells. We have therefore designed a system of orthogonal oligomerizing reagents for such purposes. Thus we provide for the use of one set of ligands and chimeric responder proteins for regulating apoptosis in the host cells, and another set for regulating the transcription of therapeutic genes.  
30 The ligands used for regulating transcription of a therapeutic or desired gene are designed or selected not cross-react and initiate apoptosis.

An exemplary chimeric cDNA has been constructed consisting of three

FKBP12 domains fused to the cytoplasmic signaling domain of the Fas antigen (Figure 19). This construct, when expressed in human Jurkat and murine D10 T cells, can be induced to dimerize by an FK1012 reagent and initiate a signaling cascade resulting in FK1012-dependent apoptosis. The LD<sub>50</sub> for FK1012A-mediated death of cells transiently transfected with MFF3E is 15 nM as determined by a loss of reporter gene activity (Figure 19; for a discussion of the assay, see legend to Figure 20). These data coincide with measurements of cell death in stably transfected cell lines. Since the stable transfectants represent a homogeneous population of cells, they have been used to ascertain that death is due to apoptosis rather than necrosis as evidenced by membrane blebbing and nucleosomal DNA fragmentation). Because the transient transfection protocol is more convenient, it has been used as an initial assay system, as is described below.

#### Example 4(C): Regulation of Programmed Cell Death with Cyclophilin-Fas Antigen Chimeras

We have also prepared a series of cyclophilin C-Fas antigen constructs and assayed their ability to induce (CsA)2-dependent apoptosis in transient expression assays (Figure 20A). In addition, (CsA)2-dependent apoptosis has been demonstrated with human Jurkat T cells stably transfected with the most active construct in the series, MC3FE (M = myristoylation domain of Src, C = cyclophilin domain, F = cytoplasmic tail of Fas, E = flu epitope tag). The cytoplasmic tail of Fas was fused either before or after 1, 2, 3, or 4 consecutive cyclophilin domains. Two control constructs were also prepared that lack the Fas domain. In this case we observed that the signaling domain functions only when placed after the dimerization domains. (The zeta chain constructs signal when placed either before or after the dimerization domains.) Both the expression levels of the eight signaling constructs, as ascertained by Western blotting, and their activities differed quantitatively (Figure 20B). The optimal system has thus far proved to be MC3FE. The LD<sub>50</sub> for (CsA)2-mediated cell death with MC3FE is ~200 nM. These data demonstrate the utility of the cyclophilin-cyclosporin interactions for regulating intracellular protein

association and illustrate an orthogonal reagent system that will not cross-react with the FKBP12-FK1012 system. Further, in this case, the data show that only dimerization and not aggregation is required for initiation of signal transduction by the Fas cytoplasmic tail.

- 5 Mutation of the N-terminal glycine of the myristoylation signal to an alanine prevents myristoylation and hence membrane localization. We have also observed that the mutated construct ( $\Delta$ MFF3E) was equally potent as an inducer of FK1012-dependent apoptosis, indicating that membrane localization is not necessary for Fas-mediated cell death.

10 **Example 5. Construction of Murine Signalling Chimeric Protein.**

The various fragments were obtained by using primers described in Figure 4. For identifying specific primer, reference should be made to Figure 4.

- 15 A cDNA fragment of approximately 1.2 kb comprising the I-E chain of the murine class II MHC receptor (*Cell*, 32, 745) was used as a source of the signal peptide, employing P#6048 and P#6049 to give a 70 bp *SacII-XhoI* fragment using PCR as described by the supplier (Promega). A second fragment was obtained using a plasmid comprising Tac (IL2 receptor chain) joined to the transmembrane and cytoplasmic domains of CD3 (*PNAS*, 88, 8905). Using P#6050 and P#6051, a 320 bp *XhoI-EcoRI* fragment was obtained  
20 by PCR comprising the transmembrane and cytoplasmic domains of CD3. These two fragments were ligated and inserted into a *SacII-EcoRI* digested pBluescript (Stratagene) to provide plasmid SPZ/KS.

- To obtain the binding domain for FK506, plasmid rhFKBP (provided by S. Schreiber, *Nature* (1990) 346, 674) was used with P#6052 and P#6053 to  
25 obtain a 340 bp *XhoI-SalI* fragment containing human FKBP12. This fragment was inserted into pBluescript digested with *XhoI* and *SalI* to provide plasmid FK12/KS, which was the source for the FKBP12 binding domain. SPZ/KS was digested with *XhoI*, phosphatased (cell intestinal alkaline phosphatase; CIP) to prevent self-annealing, and combined with a 10-fold molar excess of the *XhoI-SalI* FKBP12-containing fragment from FK12/KS. Clones were isolated that  
30 contained monomers, dimers, and trimers of FKBP12 in the correct orientation.



The clones 1FK1/KS, 1FK2/KS, and 1FK3/KS are comprised of in the direction of transcription; the signal peptide from the murine MHC class II gene I-E, a monomer, dimer or trimer, respectively, of human FKBP12, and the transmembrane and cytoplasmic portions of CD3. Lastly, the *SacII-EcoRI* fragments were excised from pBluescript using restriction enzymes and ligated into the polylinker of pBJ5 digested with *SacII* and *EcoRI* to create plasmids 1FK1/pBJ5, 1FK2/pBJ5, and 1FK3/pBJ5, respectively. See Figs. 3 and 4.

#### Example 6

##### A. Construction of Intracellular Signaling Chimera.

A myristoylation sequence from c-src was obtained from Pellman, *et al.*, *Nature* 314, 374, and joined to a complementary sequence of CD3 to provide a primer which was complementary to a sequence 3' of the transmembrane domain, namely P#8908. This primer has a *SacII* site adjacent to the 5' terminus and a *XhoI* sequence adjacent to the 3' terminus of the myristoylation sequence. The other primer P#8462 has a *SalI* recognition site 3' of the sequence complementary to the 3' terminus of CD3, a stop codon and an *EcoRI* recognition site. Using PCR, a 450 bp *SacII-EcoRI* fragment was obtained, which was comprised of the myristoylation sequence and the CD3 sequence fused in the 5' to 3' direction. This fragment was ligated into *SacII/EcoRI*-digested pBJ5(*XhoI*)(*SalI*) and cloned, resulting in plasmid MZ/pBJ5. Lastly, MZ/pBJ5 was digested with *SalI*, phosphatased, and combined with a 10-fold molar excess of the *XhoI-SalI* FKBP12-containing fragment from FK12/KS and ligated. After cloning, the plasmids comprising the desired constructs having the myristoylation sequence, CD3 and FKBP12 multimers in the 5'-3' direction were isolated and verified as having the correct structure. See Figures 2 and 4.

##### B. Construction of expression cassettes for intracellular signaling chimeras

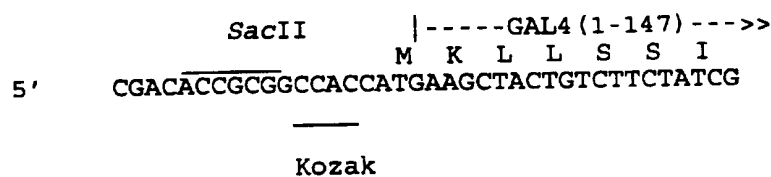
The construct MZ/pBJ5 (MZE/pBJ5) is digested with restriction enzymes *XhoI* and *SalI*, the TCR  $\zeta$  fragment is removed and the resulting vector is ligated with a 10 fold excess of a monomer, dimer, trimer or higher order multimer of FKBP12 to make MF1E, MF2E, MF3E or MF<sub>n</sub>E/pBJ5.

Active domains designed to contain compatible flanking restriction sites (i.e. XhoI and SalI) can then be cloned into the unique XhoI or SalI restriction sites of MF<sub>n</sub>E/pBJ5.

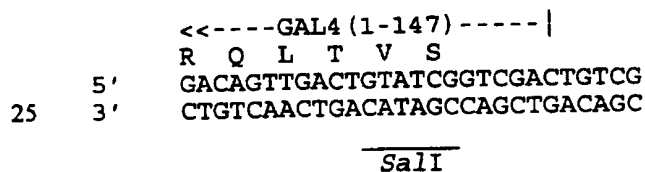
#### Example 7. Construction of Nuclear Chimera

- 5 A. GAL4 DNA binding domain - FKBP domain(s) - epitope tag. The GAL4 DNA binding domain (amino acids 1-147) was amplified by PCR using a 5' primer (#37) that contains a *Sac*II site upstream of a Kozak sequence and a translational start site, and a 3' primer (#38) that contains a *Sal*I site. The PCR product was isolated, digested with *Sac*II and *Sal*I, and ligated into pBluescript II KS (+) at the *Sac*II and *Sal*I sites, generating the construct pBS-GAL4. The construct was verified by sequencing. The *Sac*II/*Sal*I fragment from pBS-GAL4 was isolated and ligated into the IFK1/pBJ5 and IFK3/pBJ5 constructs (containing the myristoylation sequence, see Example 6) at the *Sac*II and XhoI sites, generating constructs GF1E, GF2E and GF3E.

- 15 5' end of PCR amplified product:



- 3' end of PCR amplified product:



#### B. HNF1 Dimerization/DNA Binding Domain - FKBP Domain(s) - Tag.

- 30 The HNF1a dimerization/DNA binding domain (amino acids 1-282) was amplified by PCR using a 5' primer (#39) that contains a *Sac*II site upstream of a Kozak sequence and a translational start site, and a 3' primer

(#40) that contains a *Sac*II site. The PCR product was isolated, digested with *Sac*II and *Sal*I, and ligated into pBluescript II KS (+) at the *Sac*II and *Sal*I sites, generating the construct pBS-HNF. The construct was verified by sequencing. The *Sac*II/*Sal*I fragment from pBS-HNF was isolated and ligated into the

5 IFK1/pBJ5 and IFK3/pBJ5 constructs at the *Sac*II and *Xho*I sites, generating constructs HF1E, HF2E and HF3E.

5' end of PCR amplified product:

```

          SacII      | --HNF1 (1-281) -->>
                      M V S K L S
10  5'   CGACACCGCGGCCACCATGGTTTCTAAGCTGAGC
          Kozak

```

3' end of PCR amplified product:

```

<<-----HNF1 (1-282) -----|
A F R H K L
15  5'   CCTTCGGGCACAAGTTGGTCTGACTGTCTG
      3'   GGAAGGCCGTGTTCAACCAGCTGACAGC
          SalI

```

20 C. FKBP domain(s)-VP16 transcription activation domain(s)-epitope tag.

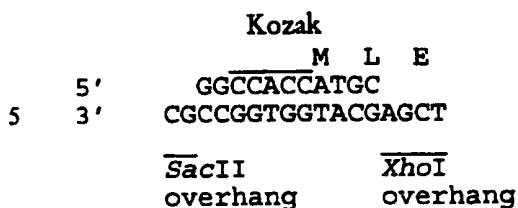
These constructs were made in three steps: (i) a construct was created from IFK3/pBJ5 in which the myristoylation sequence was replaced by a start site immediately upstream of an *Xho*I site, generating construct SF3E; (ii) a nuclear localization sequence was inserted into the *Xho*I site, generating

25 construct NF3E; (iii) the VP16 activation domain was cloned into the *Sal*I site of NF3E, generating construct NF3V1E.

(i). Complementary oligonucleotides (#45 and #46) encoding a Kozak sequence and start site flanked by *Sac*II and *Xho*I sites were annealed, phosphorylated and ligated into the *Sac*II and *Xho*I site of MF3E, generating

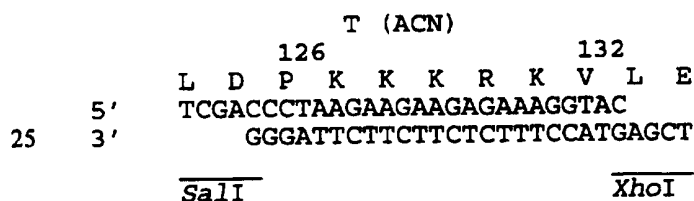
30 construct SF3E.

## Insertion of generic start site



(ii). Complementary oligonucleotides (#47 and #48) encoding the SV40 T antigen nuclear localization sequence flanked by a 5' *SacII* site and a 3' *XhoI* site were annealed, phosphorylated and ligated into the *XhoI* site of SF1E, generating the construct NF1E. The construct was verified by DNA sequencing. A construct containing the mutant or defective form of the nuclear localization sequence, in which a threonine is substituted for the lysine at position 128, was also isolated. This is designated NF1E-M. Multimers of the FKBP12 domain were obtained by isolating the FKBP12 sequence as an *XhoI/SacII* fragment from pBS-FKBP12 and ligating this fragment into NF1E linearized with *XhoI*. This resulted in the generation of the constructs NF2E and NF3E.

## 20 Insertion of NLS into generic start site



Threonine at position 128 results in a defective NLS.

(iii). The VP16 transcriptional activation domain (amino acids 413-490) was amplified by PCR using a 5' primer (#43) that contains *SacII* site and a 3' primer (#44) that contains an *XhoI* site. The PCR product was isolated, digested with *SacII* and *XhoI*, and ligated into MF3E at the *XhoI* and *SacII* sites, generating the construct MV1E. The construct was verified by sequencing. Multimerized VP16 domains were created by isolating the single VP16 sequence as a

*XhoI/SalI* fragment from MV1E and ligating this fragment into MV1E linearized with *XhoI*. Constructs MV2E, MV3E and MV4E were generated in this manner. DNA fragments encoding one or more multiple VP16 domains were isolated as *XhoI/SalI* fragments from MV1E or MV2E and ligated into NF1E linearized with *SalI*, generating the constructs NF1V1E and NF1V3E. Multimers of the FKBP12 domain were obtained by isolating the FKBP12 sequence as an *XhoI/SalI* fragment from pBS-FKBP12 and ligating this fragment into NF1V1E linearized with *XhoI*. This resulted in the generation of the constructs NF2V1E and NF3V1E.

10 5' end of PCR amplified product:

```

          SalI | --VP16(413-490) --->>
                A P P T D V
5'   CGACAGTCGACGCCCCCGACCGATGTC

```

3' end of PCR amplified product:

```

15   <<---VP16(413-490) ----|
        D E Y G G
5'   GACGAGTACGGTGGGCTCGAGTGTCTG
3'   CTGCTCATGCCACCCGAGCTCACAGC

```

20 XhoI

Oligonucleotides:

#37	38mer/0.2um/OFF	5' CGACACCGCGGCCACCATGAAGCTACTGTCTTCT
		ATCG
#38	28mer/0.2um/OFF	5' CGACAGTCGACCGATACAGTCAACTGTC
25	#39 34mer/0.2um/OFF	5' CGACACCGCGGCCACCATGGTTTCTAAGCTGAGC
	#40 28mer/0.2um/OFF	5' CGACAGTCGACCAACTTGTGCCGGAAGG
	#43 29mer/0.2um/OFF	5' CGACAGTCGACGCCCCCGACCGATGTC
	#44 26mer/0.2um/OFF	5' CGACACTCGAGCCCACCGTACTCGTC
	#45 26mer/0.2um/OFF	5' GGCCACCATGC
30	#46 18mer/0.2um/OFF	5' TCGAGCATGGTGGCCGC
	#47 27mer/0.2um/OFF	5' TCGACCCTAAGA- (C/A) -GAAGAGAAAGGTAC
	#48 27mer/0.2um/OFF	5' TCGAGTACCTTTCTCTTC- (G/T) -TCTTAGGG

#### Example 8. Demonstration of Transcriptional Induction.

Jurkat TAg cells were transfected with the indicated constructs (5 µg of each construct) by electroporation (960 µF, 250 v). After 24 hours, the cells were resuspended in fresh media and aliquoted. Half of each transfection was

- incubated with the dimeric FK506 derivative, (Example 14) at a final concentration of 1  $\mu$ M. After 12 hours, the cells were washed and cellular extracts were prepared by repeated freeze-thaw. Chloramphenicol acetyltransferase (CAT) activity was measured by standard protocols. Molecular
- 5 Cloning: A Laboratory Manual., Sambrook et al. eds. (1989) CSH Laboratory, pp. 16-59 ff. The data demonstrated CAT activity present as expected (in sample 2, with or without ligand; and in samples 5 and 6 in the presence of ligand) in 70  $\mu$ L of extract (total extract volume was 120  $\mu$ L) after incubation at 37°C for 18 hours. The samples employed in the assays are as follows:
- 10 1. G5E4TCAT (GAL4-CAT reporter plasmid)  
2. G5E4TCAT, GAL4-VP16  
3. G5E4TCAT, NF3V1E  
4. G5E4TCAT, GF2E  
5. G5E4TCAT, GF2E, NF3V1E  
15 6. G5E4TCAT, GF3E, NF3V1E

### Synthetic Chemistry Examples

As indicated elsewhere, compounds of particular interest at present as oligomerization agents have the following structure:

linker—{rbm<sub>1</sub>,rbm<sub>2</sub>, ...rbm<sub>n</sub>}.

- 20 wherein "linker" is a linker moiety such as described herein which is covalently linked to "n" (an integer from 2 to about 5, usually 2 or 3) receptor binding moieties ("rbm"s) which may be the same or different. As discussed elsewhere herein, the receptor binding moiety is a ligand (or analog thereof) for a known receptor, such as are enumerated in Section V(C), and including FK506, FK520,
- 25 rapamycin and analogs thereof which are capable of binding to an FKBP; as well as cyclosporins, tetracyclines, other antibiotics and macrolides and steroids which are capable of binding to respective receptors.

The linker is a bi- or multi-functional molecule capable of being covalently linked ("—") to two or more receptor binding moieties. Typically the linker would comprise up to about 40 atoms and may include nitrogen, oxygen and sulfur in addition to carbon and hydrogen. Illustrative linker moieties are disclosed in Section VI(A) and in the various Examples and include among others C1-C30 alkyl, alkylene, or arylalkyl groups which may be substituted or unsubstituted and may be straight-chain, branched or cyclic. For example, alkyl substituents are saturated straight-chain, cyclic or branched hydrocarbon moieties, preferably of one to about twelve carbon atoms, including methyl, ethyl, n-propyl, i-propyl, cyclopropyl, n-butyl, i-butyl, t-butyl, cyclobutyl, cyclopropylmethylene, pentyl, hexyl, heptyl, octyl and so forth, and may be optionally substituted with one or more substituents such as lower alkoxy, carboxy, amino (substituted or unsubstituted), phenyl, aryl, mercapto, halo (fluoro, chloro, bromo or iodo), azido or cyano.

These compounds may be prepared using commercially available materials and/or procedures known in the art. Engineered receptors for these compounds may be obtained as described infra. Compounds of particular interest are those which bind to a receptor with a  $K_d$  of less than  $10^{-6}$ , preferably less than about  $10^{-7}$  and even more preferably, less than  $10^{-8}$  M.

One subclass of oligomerizing agents of interest are those in which one or more of the receptor binding moieties is FK506, an FK506-type compound or a derivative thereof, wherein the receptor binding moieties are covalently attached to the linker moiety through the allyl group at C21 (using FK506 numbering) as per compound 5 or 13 in Fig 9A, or through the cyclohexyl ring (C29-C34), e.g. through the C32 hydroxyl as per compounds 8, 16, 17 in Fig 9B. Compounds of this class may be prepared by adaptation of methods disclosed herein, including in the examples which follow.

Another subclass of oligomerizing agents of interest are those in which at least one of the receptor binding moieties is FK520 or a derivative thereof, wherein the molecules of FK520 or derivatives thereof are covalently attached to the linker moiety as in FK1040A or FK 1040B in Figure 10. Compounds of this class may be prepared by adaptation of Scheme 1 in Figure 10, Scheme 2 in

Figs. 11A and 11B or Scheme 3 in Fig 12 and Fig 13.

A further subclass of oligomerizing agents of interest are those in which at least one of the receptor binding moieties is cyclosporin A or a derivative.

It should be appreciated that these and other oligomerizing agents of this invention may be homo-oligomerizing reagents (where the rbm's are the same) or hetero-oligomerizing agents (where the rbm's are different). Hetero-oligomerizing agents may be prepared by analogy to the procedures presented herein, including Scheme 3 in Figure 13 and as discussed elsewhere herein.

The following synthetic examples are intended to be illustrative.

10    **A. General Procedures.** All reactions were performed in oven-dried glassware under a positive pressure of nitrogen or argon. Air and moisture sensitive compounds were introduced via syringe or cannula through a rubber septum.

15    **B. Physical Data.** Proton magnetic resonance spectra (<sup>1</sup>H NMR) were recorded on Bruker AM-500 (500 MHz), and AM-400 (400 MHz) spectrometers. Chemical shifts are reported in ppm from tetramethylsilane using the solvent resonance as an internal standard (chloroform, 7.27 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broadened, m = multiplet), coupling constants (Hz), integration. Low and high-resolution mass spectra were obtained.

20    **C. Chromatography.** Reactions were monitored by thin layer chromatography (TLC) using E. Merck silica gel 60F glass plates (0.25 mm). Components were visualized by illumination with long wave ultraviolet light, exposed to iodine vapor, and/or by dipping in an aqueous ceric ammonium molybdate solution followed by heating. Solvents for chromatography were HPLC grade. Liquid chromatography was performed using forced flow (flash chromatography) of the indicated solvent system on E. Merck silica gel 60 (230-400 mesh).

25    **D. Solvents and Reagents.** All reagents and solvents were analytical grade and were used as received with the following exceptions. Tetrahydrofuran (THF), benzene, toluene, and diethyl ether were distilled from sodium metal  
30    benzophenone ketyl. Triethylamine and acetonitrile were distilled from calcium



hydride. Dichloromethane was distilled from phosphorous pentoxide. Dimethylformamide (DMF) was distilled from calcium hydride at reduced pressure and stored over 4A molecular sieves.

### Preparation of FK506 Derivatives

#### 5 Example 9. Hydroboration/Oxidation of FK506-TBS<sub>2</sub> (1 to 2).

The hydroboration was performed according to the procedure of Evans (Evans, *et al.*, *JACS* (1992) 114, 6679; *ibid.* (1992) 6679-6685). (See Harding, *et al.*, *Nature* (1989) 341, 758 for numbering.) A 10-mL flask was charged with 24,32-bis[(*tert*-butyldimethylsilyl)oxy]-FK506 (33.8 mg, 0.033 mmol) and  
10 [Rh(nbd)(diphos-4)]BF<sub>4</sub> (3.1 mg, 0.004 mmol, 13 mol %). The orange mixture was dissolved in toluene (2.0 mL) and the solvent was removed under reduced pressure over four hours. The flask was carefully purged with nitrogen and the orangish oil was dissolved in THF (3.0 mL, 10 mM final concentration) and cooled to 0°C with an ice water bath. Catecholborane (98 µL, 0.098 mmol,  
15 1.0 M solution in THF, 3.0 equivalents) was added via syringe and the resulting solution was stirred at 0°C for 45 min. The reaction was quenched at 0°C with 0.2 mL of THF/EtOH (1:1) followed by 0.2 mL of pH 7.0 buffer (Fisher; 0.05 M phosphate) then 0.2 mL of 30% H<sub>2</sub>O<sub>2</sub>. The solution was stirred at room temperature for at least 12 h. The solvent was removed under reduced pressure  
20 and the remaining oil was dissolved in benzene (10 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL). The phases were separated and the aqueous phase was back-extracted with benzene (2 x 10 mL). The organic phases were combined and washed once with saturated aqueous sodium bicarbonate solution (10 mL). The benzene phase was dried with  
25 MgSO<sub>4</sub>, concentrated, and subjected to flash chromatography (2:1 hexane:ethyl acetate) providing the desired primary alcohol as a clear, colorless oil (12.8 mg, 0.012 mmol, 37%).

Preparation of Mixed Carbonate (2 to 3). The preparation of the mixed carbonate was accomplished by the method of Ghosh (Ghosh, *et al.*, *Tetrahedron Lett.* (1992) 33, 2781-2784). A 10-mL flask was charged with the primary alcohol  
30

(29.2 mg, 0.0278 mmol) and benzene (4 mL). The solvent was removed under reduced pressure over 60 min. The oil was dissolved in acetonitrile (2.0 mL, 14 mM final concentration) and stirred at 20°C as triethylamine (77  $\mu$ L, 0.56 mmol) was added. N,N'-disuccinimidyl carbonate (36 mg, 0.14 mmol) was added in one portion and the solution was stirred at 20°C for 46 h. The reaction mixture was diluted with dichloromethane and washed with saturated aqueous sodium bicarbonate solution (10 mL). The phases were separated and the aqueous layer was back-extracted with dichloromethane (2 x 10 mL). The organic phases were combined and dried ( $\text{MgSO}_4$ ), concentrated, and subjected to flash chromatography (3:1 to 2:1 to 1:1 hexane:ethyl acetate). The desired mixed carbonate was isolated as a clear, colorless oil (16.8 mg, 0.014 mmol, 51%).

Dimerization of FK506 (3 to 4). A dry, 1-mL conical glass vial (Kontes Scientific Glassware) was charged with the mixed carbonate (7.3 mg, 0.0061 mmol) and acetonitrile (250  $\mu$ L, 25 mM final concentration). Triethylamine (10  $\mu$ L, 0.075 mmol) was added followed by *p*-xylylenediamine (8.3  $\mu$ L, 0.0027 mmol, 0.32 M solution in DMF). The reaction stirred 22 h at 20°C and was quenched by dilution with dichloromethane (10 mL). The solution was washed with saturated aqueous sodium bicarbonate solution (10 mL). The phases were separated and the aqueous layer was back-extracted with dichloromethane (2 x 10 mL). The organic phases were combined and dried ( $\text{MgSO}_4$ ), concentrated, and subjected to flash chromatography (3:1 to 2:1 to 1:1 hexane:ethyl acetate) providing the desired protected dimer as a clear, colorless oil (4.3 mg, 1.9  $\mu$ mol, 70%).

Deprotection of the FK506 Dimer (4 to 5). The protected dimer (3.3 mg, 1.4  $\mu$ mol) was placed in a 1.5-mL polypropylene tube fitted with a spin vane. Acetonitrile (0.5 mL, 3 mM final concentration) was added and the solution stirred at 20°C as HF (55  $\mu$ L, 48% aqueous solution; Fisher) was added. The solution was stirred 18 h at room temperature. The deprotected FK506 derivative was then partitioned between dichloromethane and saturated aqueous sodium bicarbonate in a 15-mL test tube. The tube was vortexed extensively to mix the phases and, after separation, the organic phase was removed with a pipet. The aqueous phase was back-extracted with dichloromethane (4 x 2 mL),

and the combined organic phases were dried ( $\text{MgSO}_4$ ), concentrated and subjected to flash chromatography (1:1:1 hexane:THF:ether to 1:1 THF:ether) providing the desired dimer as a clear, colorless oil (1.7 mg, 0.93  $\mu\text{mol}$ , 65%).

Following the above procedure, other monoamines and diamines may be used, such as benzylamine (14) octamethylenediamine, decamethylenediamine, etc.

**Example 10. Reduction of FK506 with L-Selectride (FK506 to 6).**

Danishefsky and coworkers have shown that the treatment of FK506 with L-Selectride provides 22-dihydro-FK506 with a boronate ester engaging the C24 and C22 hydroxyl groups (Coleman and Danishefsky, *Heterocycles* (1989) 28, 157-161; Fisher, *et al.*, *J. Org. Chem.* (1991) 56, 2900-2907).

Preparation of the Mixed Carbonate (6 to 7). A 10-mL flask was charged with 22-dihydro-FK506-*sec*-butylboronate (125.3 mg, 0.144 mmol) and acetonitrile (3.0 mL, 50 mM final concentration) and stirred at room temperature as triethylamine (200  $\mu\text{L}$ , 1.44 mmol, 10 equivalents) was added to the clear solution. N,N'-disuccinimidyl carbonate (184.0 mg, 0.719 mmol) was added in one portion, and the clear solution was stirred at room temperature for 44 h. The solution was diluted with ethyl acetate (20 mL) and washed with saturated aqueous sodium bicarbonate (10 mL) and the phases were separated. The aqueous phase was then back-extracted with ethyl acetate (2 x 10 mL), and the organic phases were combined, dried ( $\text{MgSO}_4$ ), and the resulting oil was subjected to flash chromatography (1:1 to 1:2 hexane:ethyl acetate) providing the desired mixed carbonate as a clear, colorless oil (89.0 mg, 0.088 mmol, 61%).

Dimerization of FK506 Mixed Carbonate (7 to 8). A dry, 1-mL conical glass vial (Kontes Scientific Glassware) was charged with the mixed carbonate (15.0 mg, 0.0148 mmol) and dichloromethane (500  $\mu\text{L}$ , 30 mM final concentration). The solution was stirred at room temperature as triethylamine (9  $\mu\text{L}$ , 0.067 mmol, 10 equiv.) was added followed by *p*-xylylenediamine (0.8 mg, 0.0059 mmol). The reaction stirred 16 h at 20°C and was quenched by dilution with dichloromethane (5 mL). The solution was washed with saturated aqueous sodium bicarbonate solution (5 mL). The phases were separated and the aqueous

layer was back-extracted with dichloromethane (2 x 5 mL). The organic phases were combined and dried (MgSO<sub>4</sub>), concentration, and subjected to flash chromatography (1:1 to 1:2 hexane:ethyl acetate) providing the desired dimer as a clear, colorless oil (7.4 mg, 3.8  $\mu$ mol, 65%).

- 5           Following the above procedure, other, monoamines, diamines or triamines may be used in place of the xylylenediamine, such as benzylamine (15), octylenediamine, decamethylenediamine (16), bis-p-dibenzylamine, N-methyl diethylenamine, tris-aminoethylamine (17), tris-aminopropylamine, 1,3,5-triaminomethylcyclohexane, etc.

- 10   **Example 11. Oxidative Cleavage and Reduction of FK506 (1 to 9).** The osmylation was performed according to the procedure of Kelly (VanRheenen, *et al.*, *Tetrahedron Lett.* (1976) 17, 1973-1976). The cleavage was performed according to the procedure of Danishefsky (Zell, *et al.*, *J. Org. Chem.* (1986) 51, 5032-5036). The aldehyde reduction was performed according to the procedure
- 15   of Krishnamurthy (*J. Org. Chem.*, (1981) 46, 4628-4691). A 10 mL flask was charged with 24,32-bis[*tert*-butyldimethylsilyl]oxy]-FK506 (84.4 mg, 0.082 mmol), 4-methylmorpholine N-oxide (48 mg, 0.41 mmol, 5 equiv), and THF (2.0 mL, 41 mM final concentration). Osmium tetroxide (45  $\mu$ L, 0.008 mmol, 0.1 equiv) was added via syringe. The clear, colorless solution was stirred at room
- 20   temperature for 5 hr. The reaction was then diluted with 50% aqueous methanol (1.0 mL) and sodium periodate (175 mg, 0.82 mmol, 10 equiv) was added in one portion. The cloudy mixture was stirred 40 min at room temperature, diluted with ether (10 mL), and washed with saturated aqueous sodium bicarbonate solution (5 mL). The phases were separated and the aqueous
- 25   layer was back-extracted with ether (2 x 5 mL). The combined organic layers were dried (MgSO<sub>4</sub>) and treated with solid sodium sulfite (50 mg). The organic phase was then filtered and concentrated and the oil was subjected to flash chromatography (3:1 to 2:1 hexane:ethyl acetate) providing the intermediate, unstable aldehyde (53.6 mg) as a clear, colorless oil. The aldehyde was
- 30   immediately dissolved in THF (4.0 mL) and cooled to -78°C under an atmosphere of nitrogen, and treated with lithium tris[(3-ethyl-3-

pentyl)oxy]aluminum hydride (0.60 mL, 0.082 mmol, 0.14 M solution in THF, 1.0 equiv). The clear solution was allowed to stir for 10 min at -78°C then quenched by dilution with ether (4 mL) and addition of saturated aqueous ammonium chloride (0.3 mL). The mixture was allowed to warm to room  
5 temperature and solid sodium sulfate was added to dry the solution. The mixture was then filtered and concentrated and the resulting oil was subjected to flash chromatography (2:1 hexane:ethyl acetate) giving the desired alcohol as a clear, colorless oil (39.5 mg, 0.038 mmol, 47%).

Preparation of Mixed Carbonate (9 to 10). The preparation of the mixed  
10 carbonate was accomplished by the method of Ghosh, *et al.*, *Tetrahedron Lett.* (1992) 33, 2781-2784). A 10 mL flask was charged with the primary alcohol (38.2 mg, 0.0369 mmol) and acetonitrile (2.0 mL, 10 mM final concentration) and stirred at room temperature as 2,6-lutidine (43 µL, 0.37 mmol, 10 equiv) was added. N,N'-disuccinimidyl carbonate (48 mg, 0.18 mmol) was added in one  
15 portion and the solution was stirred at room temperature for 24 h. The reaction mixture was diluted with ether (10 mL) and washed with saturated aqueous sodium bicarbonate solution (10 mL). The phases were separated and the aqueous layer was back-extracted with ether (2 x 10 mL). The organic phases were combined and dried (MgSO<sub>4</sub>), concentrated, and subjected to flash  
20 chromatography (2:1 to 1:1 hexane:ethyl acetate). The desired mixed carbonate was isolated as a clear, colorless oil (32.6 mg, 0.028 mmol, 75%).

Preparation of Benzyl Carbamate (10 to 11). A dry, 1 mL conical glass vial (Kontes Scientific Glassware) was charged with the mixed carbonate 10 (8.7 mg, 0.0074 mmol) and acetonitrile (500 µL, 15 mM final concentration).  
25 The solution was stirred at room temperature as triethylamine (10 µL, 0.074 mmol, 10 equiv) was added followed by benzylamine (1.6 µL, 0.015 mmol, 2 equiv). The reaction stirred 4 h at room temperature. The solvent was removed with a stream of dry nitrogen and the oil was directly subjected to flash chromatography (3:1 to 2:1 hexane:ethyl acetate) providing the desired protected  
30 monomer as a clear, colorless oil (6.2 mg, 5.3 µmol, 72%).

The protected monomer (6.2 mg, 5.3 µmol) was placed in a 1.5 mL polypropylene tube fitted with a spin vane. Acetonitrile (0.5 mL, 11 mM final

concentration) was added and the solution stirred at room temperature as HF (55  $\mu$ L, 48% aqueous solution; Fisher, 3.0 N final concentration) was added. The solution was stirred 18 h at room temperature. The deprotected FK506 derivative was then partitioned between dichloromethane and saturated aqueous sodium bicarbonate in a 15 mL test tube. The tube was vortexed extensively to mix the phases and, after separation, the organic phase was removed with a pipet. The aqueous phase was back-extracted with dichloromethane (4 x 2 mL), and the combined organic phases were dried ( $\text{MgSO}_4$ ), concentrated and subjected to flash chromatography (1:1 to 0:1 hexane:ethyl acetate) providing the desired deprotected benzylcarbamate as a clear, colorless oil (3.9 mg, 4.1  $\mu$ mol, 78%).

By replacing the benzylamine with a diamine such as xylylenediamine (12), hexamethylenediamine, octamethylenediamine, decamethylenediamine (13) or other diamines, dimeric compounds of the subject invention are prepared.

**Example 12. Preparation of the Mixed Carbonate of FK506 (12).** A 10-mL flask was charged with 24, 32-bis [(tert-butyldimethylsilyl)oxy]-FK506 (339.5 mg., 0.329 mmol), 4-methylmorpholine N-oxide (193 mg, 1.64 mmol, 5 equiv), water (0.20 mL) and THF (8.0 mL, 41 mM final concentration). Osmium tetroxide (0.183 mL, 0.033 mmol, 0.1 equiv, 0.18 M solution in water) was added via syringe. The clear, colorless solution was stirred at room temperature for 4.5 h. The reaction was diluted with 50% aqueous methanol (4.0 mL) and sodium periodate (700 mg, 3.29 mmol, 10 equiv) was added in one portion. The cloudy mixture was stirred 25 min at room temperature, diluted with ether (20 mL), and washed with saturated aqueous sodium bicarbonate solution (10 mL). The phases were separated and the aqueous layer was back-extracted with ether (2x10 mL). The combined organic layers were dried over  $\text{MgSO}_4$  and solid sodium sulfite (50 mg). The organic phase was then filtered and concentrated and the resulting aldehyde was immediately dissolved in THF (8.0 mL) and cooled to -78  $^{\circ}\text{C}$  under an atmosphere of nitrogen, and treated with lithium tris [(3-ethyl-3-pentyl)oxy] aluminum hydride (2.35 mL, 0.329 mmol, 0.14 M solution of THF, 1.0 equiv). The clear solution was allowed to stir for 60 min at -78  $^{\circ}\text{C}$

(monitored closely by TLC) then quenched at -78 °C by dilution with ether (5 mL) and addition of saturated aqueous ammonium chloride (0.3 mL). The mixture was allowed to warm to room temperature and solid sodium sulfate was added to dry the solution. The mixture was stirred 20 min, filtered, concentrated, and the resulting oil was immediately dissolved in acetonitrile (10 mL). To the solution of the resulting primary alcohol in CH<sub>3</sub>CN was added 2,6-lutidine (0.380 mL, 3.3. mmol, 10 equiv) and N,N'-disuccinimidyl carbonate (420 mg, 1.65 mmol, 5 equiv). The heterogenous mixture was stirred at room temperature for 19 h, at which time the solution was diluted with ether (30 mL) and washed with saturated aqueous sodium bicarbonate (20 mL). The aqueous phase was back-extracted with ether (2x10 mL). The organic phases were combined and dried (MgSO<sub>4</sub>), concentrated, and subjected to flash chromatography (3:1 to 2:1 to 1:1 hexane/ethyl acetate). The desired mixed carbonate 12 was isolated as a clear, colorless oil (217 mg, 0.184 mmol, 56% overall for 4 steps).

**Example 13. Preparation of 24, 24', 32, 32'-tetrakis [(tertbutyl-dimethylsilyl)oxy]-FK1012-A (p-xylylenediamine bridge).**

A dry, 1-mL conical glass vial was charged with the mixed carbonate (23.9 mg, 0.0203 mmol) and acetonitrile (500 µL, 41 mM final concentration). Triethylamine (28 µL, 0.20 mmol, 10 equiv) was added followed by p-xylylenediamine (46 µL, 0.0101 mmol, 0.22 M solution in DMF). The reaction stirred 18 h at room temperature, the solvent was removed with a stream of dry nitrogen, and the oil was directly subjected to flash chromatography (3:1 to 2:1 to 1:1 hexane/ethyl acetate) affording the desired protected dimer as a clear, colorless oil (11.9 mg, 5.3 µmol, 52%).

**Example 14. Preparation of FK1012-A (p-xylylenediamine bridge) (13).** The protected dimer (11.0 mg, 4.9 µmol) was placed in a 1.5-mL polypropylene tube fitted with a spin vane. Acetonitrile (0.50 mL, 10 mM final concentration) was added, and the solution stirred at 20 °C as HF (55 µL, 48% aqueous solution; Fisher, 3.0 N final concentration) was added. The solution was stirred 16h at

room temperature. The deprotected FK506 derivative was then partitioned between dichloromethane and saturated aqueous sodium bicarbonate in a 15-mL test tube. The tube was vortexed extensively to mix the phases and, after separation, the organic phase was removed with a pipet. The aqueous phase was back-extracted with dichloromethane (4x2 mL), and the combined organic phases were dried (MgSO<sub>4</sub>), concentrated and subjected to flash chromatography (1:1:1 hexane/THF/ether to 1:1 THF/ether) providing FK1012-A as a clear, colorless oil (5.5 mg, 3.0  $\mu$ mol, 63%).

**Example 15. Preparation of 24, 24', 32, 32'-tetrakis[(ter-butyl)dimethylsilyl]oxy]-FK1012-B (diaminodecane bridge).** A dry, 1-mL conical glass vial was charged with the mixed carbonate (53.3 mg, 0.0453 mmol) and acetonitrile (2.0 mL, 11 mM final concentration). Triethylamine (16  $\mu$ L, 0.11 mmol, 5 equiv) was added followed by diaminodecane (61  $\mu$ L, 0.0226 mmol, 0.37 M solution in DMF). The reaction stirred 12 h at room temperature, the solvent was removed with a stream of dry nitrogen, and the oil was directly subjected to flash chromatography (3:1 to 2:1 to 1:1 hexane/ethyl acetate) affording the desired protected dimer as a clear, colorless oil (18.0 mg, 7.8  $\mu$ mol, 35%).

**Example 16. Preparation of FK1012-B (diaminodecane -1,10 bridge) (14).** The protected dimer (18.0 mg, 7.8  $\mu$ mol) was placed in a 1.5-mL polypropylene tube fitted with a stirring flea. Acetonitrile (0.45 mL, 16 mM final concentration) was added, and the solution stirred at room temperature as HF (55  $\mu$ L, 48% aqueous solution; Fisher, 3.6 N final concentration) was added. The solution was stirred 17 h at 23 °C. The product FK1012-B was then partitioned between dichloromethane and saturated aqueous sodium bicarbonate in a 15-mL test tube. The tube was vortexed extensively to mix the phases and, after separation, the organic phase was removed with a pipet. The aqueous phase was back-extracted with dichloromethane (4x2 mL), and the combined organic phases were dried (MgSO<sub>4</sub>), concentrated and subjected to flash chromatography (100% ethyl acetate to 20:1 ethyl acetate/methanol) affording FK1012-B as a



clear, colorless oil (5.3 mg, 2.9  $\mu$ mol, 37%).

**Example 17. Preparation of 24, 24', 32, 32'-tetrakis[(tert-butyl)dimethylsilyl]oxy]-FK1012-C (bis-p-aminomethylbenzoyl diaminodecane bridge).** A dry 25-mL tear-shaped flask was charged with the diamine linker (15.1 mg, 0.0344 mmol) and 1.0 mL of DMF. In a separate flask, the mixed carbonate and triethylamine (0.100 mL, 0.700 mmol, 20 equiv) were dissolved in 2.0 mL of dichloromethane then added slowly (4x0.50 mL) to the stirring solution of bis-p-aminomethylbenzoyl, diaminodecane -1,10. The flask containing the mixed carbonate 12 was washed with dichloromethane (2x0.50 mL) to ensure complete transfer of the mixed carbonate 12. The reaction stirred 16 h at 23 °C, the solvent was removed with a stream of dry nitrogen, and the oil was directly subjected to flash chromatography (1:1 to 1:2 hexane/ethyl acetate) to afford the desired protected dimer as a clear, colorless oil (29.6 mg, 11.5  $\mu$ mol, 34%).

**Example 18. Preparation of FK1012-C (15).** The protected dimer (29.6 mg, 11.5  $\mu$ mol) (17) was placed in a 1.5-mL polypropylene tube fitted with a stirring flea. Acetonitrile (0.45 mL, 23 mM final concentration) was added, and the solution stirred at room temperature as HF (55  $\mu$ L, 48% aqueous solution; Fisher, 3.6 N final concentration) was added. The solution was stirred 17 h at room temperature. The desired symmetrical dimer was then partitioned between dichloromethane and saturated aqueous sodium bicarbonate in a 15-mL test tube. The tube was vortexed extensively to mix the phases and, after separation, the organic phase was removed with a pipet. The aqueous phase was back-extracted with dichloromethane (4x2 mL), and the combined organic phases were dried ( $\text{MgSO}_4$ ), concentrated and subjected to flash chromatography (100% ethyl acetate to 15:1 ethyl acetate/methanol) affording FK1012-C as a clear, colorless oil (11.5 mg, 5.5  $\mu$ mol, 47%).

**Preparation of CsA Derivatives**

**Example 19. MeBmt(OAc)-OH<sup>1</sup>CsA (2).** MeBmt(OAc)-OAc<sup>1</sup>-CsA (1) (161 mg, 124 mmol) (see Eberle and Nuninger, *J. Org. Chem.* (1992) 57, 2689) was dissolved in Methanol (10 mL). KOH (196 mg) was dissolved in water (8 mL). 297  $\mu$ L of the KOH solution (.130 mmol, 1.05 eq.) was added to the solution of (1) in MeOH. This new solution was stirred at room temperature under an inert atmosphere for 4 hours at which time the reaction was quenched with acetic acid (2 mL). The reaction mixture was purified by reversed phase HPLC using a 5 cm x 25 cm, 12  $\mu$ , 100 A, C18 column at 70°C eluting with 70% acetonitrile/H<sub>2</sub>O containing 0.1% (v/v) Trifluoroacetic acid to give 112 mg (72%) of the desired monoacetate (2).

MeBmt(OAc)-OCOIm<sup>1</sup>CsA (3). MeBmt(OAc)-OH<sup>1</sup>-CsA (2) (57 mg, 45.5  $\mu$ mol) and carbonyldiimidazole (15 mg, 2 eq., 91  $\mu$ mol.) were transferred into a 50 mL round bottom flask and dissolved in dry THF (6 mL). Diisopropylethylamine (32  $\mu$ L, 4 eq., 182  $\mu$ mol) was added and then the solvent was removed on a rotary evaporator at room temperature. The residue was purified by flash chromatography on silica gel using ethyl acetate as eluent to give 45 mg (73%) of the desired carbamate (3).

Tris-(2-aminoethyl)amine CsA Trimer Triacetate (6). MeBmt(OAc)-OCOIm<sup>1</sup>-CsA (3) (7.5 mg, 5.54  $\mu$ mol, 3.1 eq.) was dissolved in THF (100  $\mu$ L). Diisopropylethylamine (62  $\mu$ L, 5 eq., 8.93  $\mu$ mol of a solution containing 100  $\mu$ L of amine in 4 mL THF) was added followed by tris(2-aminoethyl)amine (26  $\mu$ L, 1.79  $\mu$ mol, 1 eq. of a solution containing 101 mg of tris-amine in 10 mL THF). This solution was allowed to stir under N<sub>2</sub> atmosphere for 5 days. The reaction mix was evaporated and then purified by flash chromatography on silica gel using 0-5% methanol in chloroform to give 4.1 mg of desired product (6).

**Example 20. Diaminodecane CsA Dimer (8).** Solid Na metal (200 mg, excess) was reacted with dry methanol (10 mL) at 0°C. Diaminodecane CsA Dimer Diacetate (5) (4.0 mg) was dissolved in MeOH (5 mL). 2.5 mL of the NaOMe solution was added to the solution of (5). After 2.5 hours of stirring at room temperature under an inert atmosphere, the solution was quenched with acetic acid (2 mL) and the product was purified by reversed phase HPLC using a

5 mm x 25 mm, 12  $\mu$ , 100 Å, C18 column at 70°C eluting with 70-95% acetonitrile/H<sub>2</sub>O over 20 minutes containing 0.1% (v/v) Trifluoroacetic acid to give 2.5 mg (60%) of the desired diol.

The daminodecane CsA Dimer Diacetate (5) was prepared by replacing the tris(2-aminoethyl)amine with 0.45 eq. of 1,10-daminodecane.

**Example 21. p-Xylylenediamine CsA Dimer (4).**

The p-xylylene diamine CsA Dimer (4) was prepared by replacing the tris(2-aminoethyl)amino with 0.45 eq. of p-xylylene diamine.

Following procedures described in the literature other derivatives of cyclophilin are prepared by linking at a site other than the 1(MeBmt 1) site.

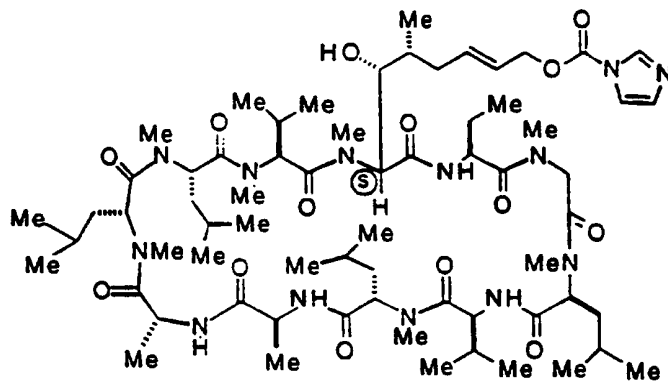
Position 8 D-isomer analogues are produced by feeding the producing organism with the D-amino analogue to obtain incorporation specifically at that site. See Patchett, *et al.*, *J. Antibiotics* (1992) 45, 943 ( $\beta$ -MeSO)D-Ala<sup>8</sup>-CsA); Traber, *et al.*, *ibid.* (1989) 42, 591). The position 3 analogues are prepared by poly-lithiation/alkylation of CsA, specifically at the -carbon of Sac3. See Wenger, *Transplant Proceeding* (1986) 18, 213, supp. 5 (for cyclophilin binding and activity profiles, particularly D-MePhe<sup>3</sup>-CsA); Seebach, U.S. Patent No. 4,703,033, issued October 27, 1987 (for preparation of derivatives).

Instead of cyclosporin A, following the above-described procedures, other naturally-occurring variants of CsA may be multimerized for use in the subject invention.

**Example 21A. Alternative synthesis for CsA dimer.**

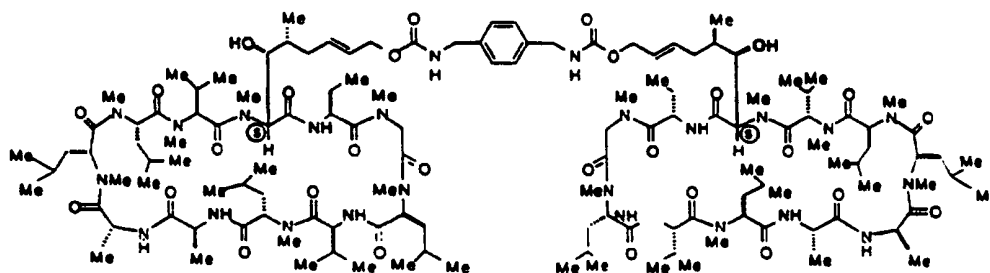
**MeBmt(OH)- $\eta$ -OCOIm<sup>1</sup>-CsA**

MeBmt(OH)- $\eta$ -OH<sup>1</sup>-CsA (38 mg, 31  $\mu$ mol, 1218.6 g/mol) and carbonyl-diimidazole (20 mg, 4eq., 124  $\mu$ mol, 162.15 g/mol) were transferred into a 10 mL round bottom flask and dissolved in dry THF (2 mL). Diisopropylethylamine (22 mL, 4 eq., 125  $\mu$ mol, 129.25 g/mol) was added and then the solvent was removed on a rotary evaporator at room temperature. The residue was purified by flash chromatography on silica gel using 0-20% acetone in ethyl acetate as eluent to give 32mg (78% yield) of a white solid.



**(CsA)<sub>2</sub> xylylenediamine CsA dimer**

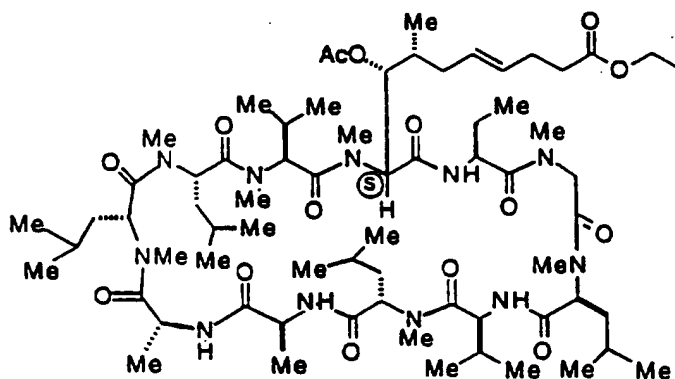
MeBmt(OH)- $\eta$ -OCOIm<sup>1</sup>-CsA (12.5 mg, 9.52 mmol, 1312.7 g/mol) was dissolved in DCM (200mL). To this solution was added 22ml (0.5eq., 4.75 mmol) of a solution of xylylene diamine (14.7 mg, 136.2g/mol) in DMSO (0.5 mL) and the reaction mixture was stirred for 72 hours at room temperature under a nitrogen atmosphere concentrating slowly. The reaction was diluted with acetonitrile (2 mL) filtered through glass wool and purified by reverse phase HPLC (Beckman C18, 10m, 100A, 1cm x 25cm, 5mL/min, 50 to 90% ACN/H<sub>2</sub>O(+0.1%TFA) over 30 minutes, 70°C) to give 6.1 mg (49% yield) of a white solid.



**Example 21B. Synthesis of a FK506-CsA dimer - MeBmt(OAc)- $\eta$ -CH<sub>2</sub>COOEt-CsA**

MeBmt(OAc)- $\eta$ -Br<sup>1</sup>-CsA (26 mg, ~80% pure, 15.7 mmol, 1323.57 g/mol) was dissolved in THF (500 mL). This solution was added by syringe pump over 15 hours to a THF solution of the magnesium enolate of ethyl hydrogen malonate (excess) prepared by the addition of iPrMgCl (2.15 mL, 2.34 M in ether) to a 0°C solution of ethyl hydrogen malonate (Lancaster, 2.5 mmol, 332 mg, 132.12 g/mol) in THF (4.7 mL) followed by warming to room temperature. The reaction mixture was quenched with 1N HCL (50 mL) and extracted with ethyl acetate (2 x 50 mL). The organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated.

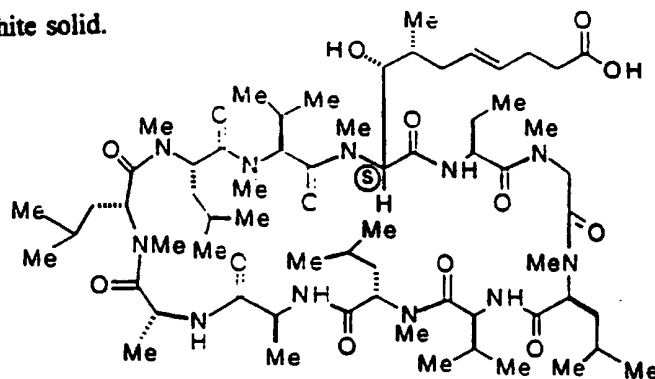
The crude product was dissolved in DMF (1 mL). Et<sub>4</sub>NOAc.4H<sub>2</sub>O (150 mg. excess) was added and the mixture was heated at 90°C for 2 hours. The reaction mixture was cooled to room temperature, diluted with H<sub>2</sub>O (50 mL) and extracted with ether (2 x 50 mL). The combined organics were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated. The residue was purified by flash chromatography on silica gel, eluting with 75-100% ethyl acetate/hexanes to give 11.4 mg (55%) of a white solid.



**MeBmt(OH)- $\eta$ -CH<sub>2</sub>COOH<sup>1</sup>-CsA**

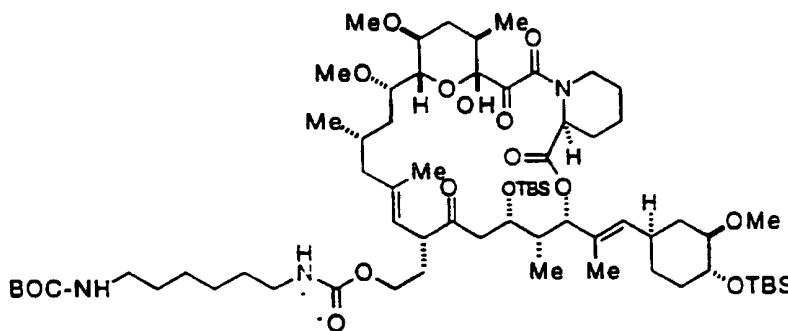
MeBmt(OAc)- $\eta$ -CH<sub>2</sub>COOEt<sup>1</sup>-CsA (11.0 mg, 8.27 mmol, 1330.76 g/mol) was dissolved in MeOH (2 mL) and added to a solution of NaOMe (1.30 M in MeOH, 10 mL). The reaction mixture was stirred at room temperature under a nitrogen atmosphere for 5 hours at which time H<sub>2</sub>O (2 mL) was added and the mixture was stirred for another 2 hours. The reaction was quenched with glacial

acetic acid (1 mL), filtered through glass wool and purified by reverse phase HPLC (Rainin C18 dynamax, 5m, 300A, 21.4 mm x 250 mm, 20 mL/min, 50 to 90% ACN/H<sub>2</sub>O(+0.1%TFA) over 30 minutes, 70°C) to give 5.5 mg (53% yield) of a white solid.



5 **bis-TBS-N-(6-(Boc-amino)hexyl) FK506 carbamate**

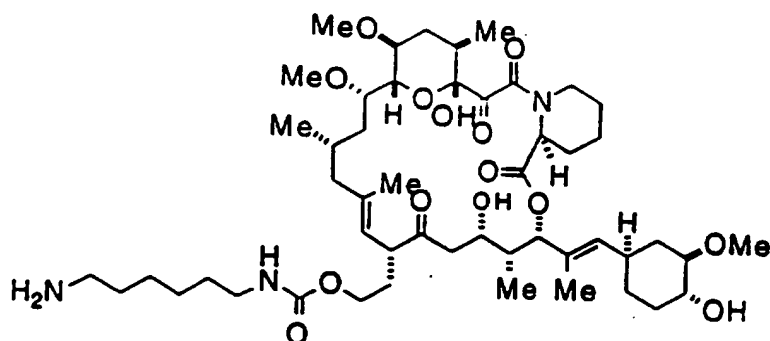
bis-TBS-FK506 succinimidyl carbonate (also a precursor to (tbs)4-FK1012) (5.8 mg, 1177.62 g/mol, 4.93 mmol) was dissolved in DCM. To this was added N-Boc-1,6-diaminohexane (7.25 mg, excess). After stirring for 10 min at room temperature the reaction mixture was evaporated and the product purified by flash chromatography eluting with 10 to 40% ethyl acetate/hexanes to provide 5.9 mg (94 % yield).



15 **N-(6-amino)hexyl FK506 carbamate**

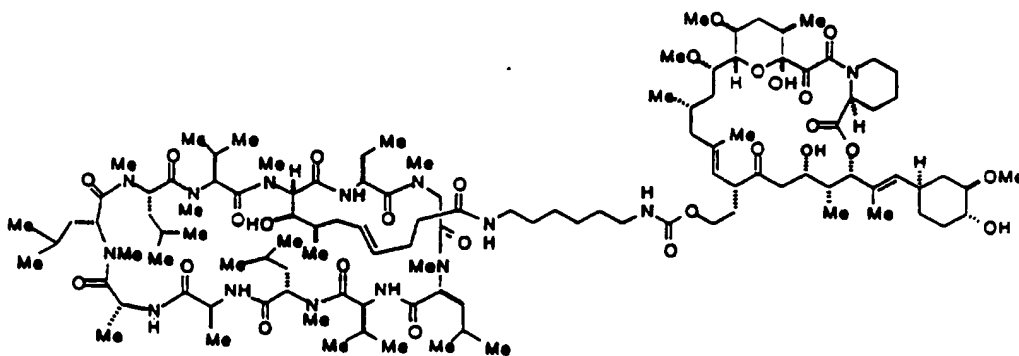
bis-TBS-N-(6-(Boc-amino)hexyl) FK506 carbamate (5.9 mg, 1278.88 g/mol, 4.61 mmol) was transferred to a polypropylene tube in ACN (700 ml) followed by aqueous HF (49%, 100mL). The reaction was complete after six hours at room temperature and was quenched by the slow addition of a saturated solution of NaHCO<sub>3</sub>. The mixture was diluted with saturated NaHCO<sub>3</sub> (4mL),

H<sub>2</sub>O (4mL) and extracted with DCM (3 x 10 mL). The combined organic phases were dried with MgSO<sub>4</sub>, filtered and evaporated to give 3.6 mg (82% yield) of crude product.



### FKCsA

- 5 MeBmt(OH)- $\eta$ -CH<sub>2</sub>COOH<sup>1</sup>-CsA (2.86 mg, 2.27 mmol, 1260.66 g/mol) and N-(6-aminohexyl) FK506 carbamate (crude, 2.16 mg, 2.28 mmol, 949.21 g/mol) were dissolved in DCM (900mL). To this solution was added 127ml (3.0eq., 6.8 mmol) of a solution of BOP (11.9 mg, 442.5 g/mol) in DCM (500 mL), followed by 45 mL (2.25 eq., 5.1 mmol) of a solution of diisopropylethyl amine (20 mL, 10  
d=0.74 2129.25 g/mol) in DCM (1.0 mL). Finally DMF (40 mL) was added and the reaction mixture was evaporated slowly at room temperature under a stream of nitrogen over 12 hours. The reaction mixture was diluted with acetonitrile (1 mL) filtered through glass wool and purified by reverse phase HPLC (Beckman C18, 1cm x 25cm, 5mL/min, 50 to 90% ACN/H<sub>2</sub>O over 25 minutes, 50°C) to  
15 give 2.4 mg (48% yield) of a white solid.



**Example 22. (A) Structure-Based Design and Synthesis of FK1012-"Bump" Compounds and FKBP12s with Compensatory Mutations**

Substituents at C9 and C10 of FK506, which can be and have been accessed by synthesis, clash with a distinct set of FKBP12 sidechain residues. Thus, one class of mutant receptors for such ligands should contain distinct modifications, one creating a compensatory hole for the C10 substituent and one for the C9 substituent. Carbon 10 was selectively modified to have either an N-acetyl or N-formyl group projecting from the carbon (vs. a hydroxyl group in FK506). The binding properties of these derivatives clearly reveal that these C10 bumps effectively abrogate binding to the native FKBP12. Figure 21 depicts syntheses of FK506-type moieties containing additional C9 bumps. By assembling such ligands with linker moieties of this invention one can construct HED and HOD (and antagonist) reagents for chimeric proteins containing corresponding binding domains bearing compensatory mutations. An illustrative HED reagent is depicted in Figure 21 that contains modifications at C9 and C10'.

This invention thus encompasses a class of FK506-type compounds comprising an FK506-type moiety which contains, at one or both of C9 and C10, a functional group comprising -OR, -R, -(CO)OR, -NH(CO)H or -NH(CO)R, where R is substituted or unsubstituted, alkyl or arylalkyl which may be straightchain, branched or cyclic, including substituted or unsubstituted peroxides, and carbonates. "FK506-type moieties" include FK506, FK520 and synthetic or naturally occurring variants, analogs and derivatives thereof (including rapamycin) which retain at least the (substituted or unsubstituted) C2 through C15 portion of the ring structure of FK506 and are capable of binding with a natural or modified FKBP, preferably with a  $K_d$  value below about  $10^{-6}$  M.

This invention further encompasses homo- and hetero-dimers and higher order oligomers containing one or more of such FK506-type compounds covalently linked to a linker moiety of this invention. Monomers of these FK506-type compounds are also of interest, whether or not covalently attached to a linker moiety or otherwise modified without abolishing their binding



affinity for the corresponding FKBP. Such monomeric compounds may be used as oligomerization antagonist reagents, i.e., as antagonists for oligomerizing reagents based on a like FK506-type compound. Preferably the compounds and oligomers comprising them in accordance with this invention  
5 bind to natural, or preferably mutant, FKBP's with an affinity at least 0.1% and preferably at least about 1% and even more preferably at least about 10% as great as the affinity of FK506 for FKBP12. See e.g. Holt et al., *infra*.

Receptor domains for these and other ligands of this invention may be obtained by structure-based, site-directed or random mutagenesis methods. We  
10 contemplate a family of FKBP12 moieties which contain Val., Ala, Gly, Met or other small amino acids in place of one or more of Tyr26, Phe36, Asp37, Tyr82 and Phe99 as receptor domains for FK506-type and FK520-type ligands containing modifications at C9 and/or C10. In particular, we contemplate using FKBP's with small replacements such as Gly or Ala for Asp37 in  
15 conjunction with FK506-type and FK520-type ligands containing substituents at C10 (e.g., -NHCOR, where R is alkyl, preferably lower alkyl such as methyl for example; or -NHCHO), and FKBP's with small replacements such as Gly or Ala for Phe36, Phe99 and Tyr26 in conjunction with FK506-type and FK520-type ligands containing replacements at C9 (e.g., oxalazines or imines).

20 Site-directed mutagenesis may be conducted using the megaprimer mutagenesis protocol (see e.g., Sakar and Sommer, *BioTechniques* 8 4 (1990): 404-407). cDNA sequencing is performed with the Sequenase kit. Expression of mutant FKBP12s may be carried out in the plasmid pHN1<sup>+</sup> in the *E. coli* strain XA90 since many FKBP12 mutants have been expressed in this system  
25 efficiently. Mutant proteins may be conveniently purified by fractionation over DE52 anion exchange resin followed by size exclusion on Sepharose as described elsewhere. See e.g. Aldape et al., *J. Biol. Chem.* 267 23 (1992): 16029-32 and Park et al., *J. Biol. Chem.* 267 5 (1992): 3316-3324. Binding constants may be readily determined by one of two methods. If the mutant FKBP's  
30 maintain sufficient rotamase activity, the standard rotamase assay may be utilized. See e.g., Galat et al., *Biochemistry* 31 (1992): 2427-2434. Otherwise, the mutant FKBP12s may be subjected to a binding assay using LH20 resin and

radiolabeled  $^3\text{H}_2$ -dihydroFK506 and  $^3\text{H}_2$ -dihydroCsA that we have used previously with FKBP<sub>s</sub> and cyclophilins. Bierer et al., *Proc. Natl. Acad. Sci. U.S.A.* 87 4 (1993): 555-69.

#### (B) Selection of Compensatory Mutations in FKBP12 for Bump-FK506<sub>s</sub>

##### 5 Using the Yeast Two-Hybrid System

One approach to obtaining variants of receptor proteins or domains, including of FKBP12, is the yeast "two-hybrid" or "interaction trap" system. The two-hybrid system has been used to detect proteins that interact with each other. A "bait" fusion protein consisting of a target protein fused to a  
10 transcriptional activation domain is co-expressed with a cDNA library of potential "hooks" fused to a DNA-binding domain. A protein-protein (bait-hook) interaction is detected by the appearance of a reporter gene product whose synthesis requires the joining of the DNA-binding and activation domains. The yeast two-hybrid system mentioned here was originally  
15 developed by Elledge and co-workers. Durfee et al., *Genes & Development* 7 4 (1993): 555-69 and Harper et al., *Cell* 75 4 (1993): 805-816.

Since the two-hybrid system per se cannot provide insights into receptor-ligand interactions involving small molecule, organic ligands, we have developed a new, FK1012-inducible transcriptional activation system (discussed  
20 below). Using that system one may extend the two hybrid system so that small molecules (e.g., FK506<sub>s</sub> or FK1012<sub>s</sub> or FK506-type molecules of this invention) can be investigated. One first generates a cDNA library of mutant FKBP<sub>s</sub> (the hooks) with mutations that are regionally localized to sites that surround C9 and C10 of FK506. For the bait, two different strategies may be pursued.  
25 The first uses the ability of FK506 to bind to FKBP12 and create a composite surface that binds to calcineurin. The sequence-specific transcriptional activator is thus comprised of: DNA-binding domain-mutant FKBP12-bump-FK506-calcineurin A-activation domain (where — refers to a noncovalent binding interaction). The second strategy uses the ability of FK1012<sub>s</sub> to bind two  
30 FKBP<sub>s</sub> simultaneously. A HED version of an FK1012 may be used to screen for the following ensemble: DNA-binding domain-mutant FKBP12-bump-

FK506—normal FK506—wildtype FKBP12—activation domain.

1. *Calcineurin-GAL4 activation domain fusion as a bait:* A derivative of pSE1107 that contains the GAL4 activation domain and calcineurin A subunit fusion construct has been constructed. Its ability to act as a bait in the proposed manner has been verified by studies using the two-hybrid system to map out calcineurin's FKBP-FK506 binding site.
2. *hFKBP12-GAL4 activation domain fusion as a bait:* hFKBP12 cDNA may be excised as an EcoRI-HindIII fragment that covers the entire open reading frame, blunt-ended and ligated to the blunt-ended Xho I site of pSE1107 to generate the full-length hFKBP-GAL4 activation domain protein fusion.
3. *Mutant hFKBP12 cDNA libraries* hFKBP12 may be digested with EcoRI and HindIII, blunted and cloned into pAS1 (Durfee et al., *supra*) that has been cut with NcoI and blunted. This plasmid is further digested with NdeI to eliminate the NdeI fragment between the NdeI site in the polylinker sequence of pAS1 and the 5' end of hFKBP12 and religated. This generated the hFKBP12-GAL4 DNA binding domain protein fusion. hFKBP was reamplified with primers #11206 and #11210, Primer Table:

```

11206
5'ndFK: 5'-GGAATTC NdeI CAT ATG GGC GTG CAG G-3'
          H M G V Q

11207
35mFK37: 5'-CTGTC SmaI CCG GGA NNN NNN NNN TTT CTT TCC ATC TTC AAG C-:
          R S X X X K K G D E L

11208
35mFK27: 5'-CTGTC SmaI CCG GGA GGA ATC AAA TTT CTT TCC ATC TTC AAG CA'
          R S S D F K K G D E L M
          NNN NNN NNN GTG CAC CAC GCA GG-3'
          X X X H V V C

11209
38mFK98: 5'-CGC BamHI GGA TCC TCA TTC CAG TTT TAG AAG CTC CAC ATC NNN
          END E L K L L E V D X
          NNN NNN AGT GGC ATG TGG-3'
          X X T A H P

11210
38mFK: 5'-CGC BamHI GGA TCC TCA TTC CAG TTT TAG AAG C-3'
          END E L K L L
  
```

Primer Table: Primers used in the construction of a regionally localized hFKBP12 cDNA library for use in screening for compensatory mutations.

Mutant hFKBP12 cDNA fragments were then prepared using the primers listed below that contain randomized mutant sequences of hFKBP at defined positions by the polymerase chain reaction, and were inserted into the GAL4 DNA binding domain-hFKBP(NdeI/BamHI) construct.

- 5     4. *Yeast strain* *S. cerevisiae* Y153 carries two selectable marker genes (*his3*/ $\beta$ -galactosidase) that are integrated into the genome and are driven by GAL4 promoters. (Durfee, *supra*.)

Using Calcineurin-GAL4 Activation Domain as Bait The FKBP12-FK506 complex binds with high affinity to calcineurin, a type 2B protein phosphatase.  
10 Since we use C9- or C10-bumped ligands to serve as a bridge in the two-hybrid system, only those FKBP12s from the cDNA library that contain a compensatory mutation generate a transcriptional activator. For convenience, one may prepare at least three distinct libraries (using primers 11207-11209, Primer Table) that will each contain 8,000 mutant FKBP12s. Randomized sites were  
15 chosen by inspecting the FKBP12-FK506 structure, which suggested clusters of residues whose mutation might allow binding of the offending C9 or C10 substituents on bumped FK506s. The libraries are then individually screened using both C9- and C10-bumped FK506s. The interaction between a bumped-FK506 and a compensatory hFKBP12 mutant can be detected by the ability of  
20 host yeast to grow on *his* drop-out medium and by the expression of  $\beta$ -galactosidase gene. Since this selection is dependent on the presence of the bumped-FK506, false positives can be eliminated by subtractive screening with replica plates that are supplemented with or without the bumped-FK506 ligands.

- 25     Using hFKBP12-GAL4 Activation Domain as Bait Using the calcineurin A-GAL4 activation domain to screen hFKBP12 mutant cDNA libraries is a simple way to identify compensatory mutations on FKBP12. However, mutations that allow bumped-FK506s to bind hFKBP12 may disrupt the interaction between the mutant FKBP12—bumped-FK506 complex and calcineurin. If the

initial screening with calcineurin as a bait fails, the wild type hFKBP12-GAL4 activation domain will instead be used. An FK1012 HED reagent consisting of: native-FK506-bumped-FK506 (Figure 16) may be synthesized and used as a hook. The FK506 moiety of the FK1012 can bind the FKBP12-GAL4  
5 activation domain. An interaction between the bumped-FK506 moiety of the FK1012 and a compensatory mutant of FKBP12 will allow host yeast to grow on his drop-out medium and to express  $\beta$ -galactosidase. In this way, the selection is based solely on the ability of hFKBP12 mutant to interact with the bumped-FK506. The same subtractive screening strategy can be used to  
10 eliminate false positives.

In addition to the in vitro binding assays discussed earlier, an in vivo assay may be used to determine the binding affinity of the bumped-FK506s to the compensatory hFKBP12 mutants. In the yeast two-hybrid system,  $\beta$ -gal activity is determined by the degree of interaction between the "bait" and the  
15 "prey". Thus, the affinity between the bumped-FK506 and the compensatory FKBP12 mutants can be estimated by the corresponding  $\beta$ -galactosidase activities produced by host yeasts at different HED (native-FK506-bumped-FK506) concentrations.

Using the same strategy, additional randomized mutant FKBP12 cDNA  
20 libraries may be created in other bump-contact residues with low-affinity compensatory FKBP12 mutants as templates and may be screened similarly.

#### Phage Display Screening for High-Affinity Compensatory FKBP Mutations

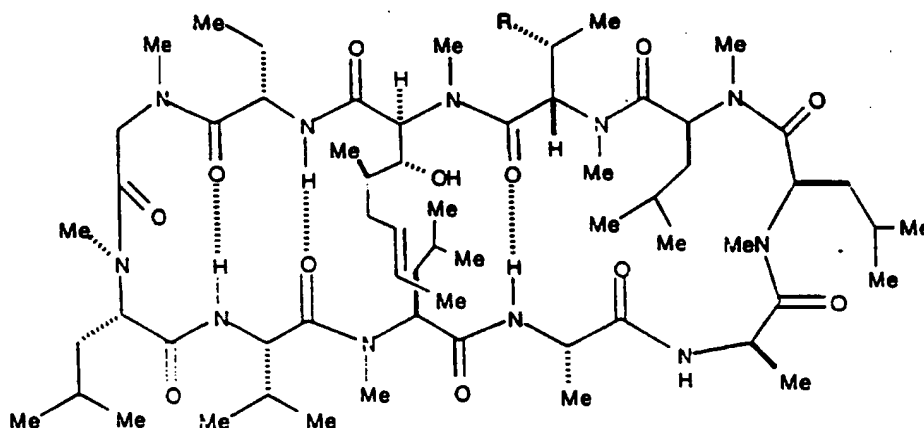
Some high-affinity hFKBP12 mutants for bump-FK506 may contain several combined point mutations at discrete regions of the protein. The size of the  
25 library that contains appropriate combined mutations can be too large for the yeast two-hybrid system's capacity (e.g.,  $> 10^8$  mutations). The use of bacteriophage as a vehicle for exposing whole functional proteins should greatly enhance the capability for screening a large numbers of mutations. See e.g. Bass et al., Proteins: Structure, Function & Genetics 8 4 (1990): 309-14; McCafferty et al., Nature 348 6301 (1990): 552-4; and Hoogenboom, Nucl. Acids Res. 19  
30 15 (1991): 4133-7. If the desired high-affinity compensatory mutants is not be

identified with the yeast two-hybrid system, a large number of combined mutations can be created on hFKBP12 with a phage vector as a carrier. The mutant hFKBP12 fusion phages can be screened with bumped-FK506-Sepharose as an affinity matrix, which can be synthesized in analogy to our original  
5 FK506-based affinity matrices. Fretz et al., J Am Chem Soc 113 4 (1991): 1409-1411. Repeated rounds of binding and phage amplification should lead to the identification of high-affinity compensatory mutants.

### (C) Synthesis of "Bumped (CsA)2s": Modification of MeVal(11)CsA

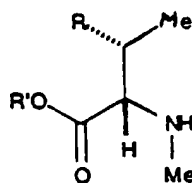
As detailed above, we have demonstrated the feasibility of using  
10 cyclophilin as a dimerization domain and (CsA)2 as a HOD reagent in the context of the cell death signaling pathway. However, to further optimize the cellular activity of the (CsA)2 reagent one may rely upon similar strategies as described with FK1012s. Thus, modified (bumped) CsA-based oligomerizing reagents should be preferred in applications where it is particularly desirable for  
15 the reagent to be able to differentiate its target, the artificial protein constructs, from endogenous cyclophilins.

One class of modified CsA derivatives of this invention are CsA analogs in which (a) NMeVal11 is replaced with NMePhe (which may be substituted or unsubstituted) or NMeThr (which may be unsubstituted or substituted on the  
20 threonine betahydroxyl group) or (b) the *pro-S* methyl group of NMeVal11 is replaced with a bulky group of at least 2 carbon atoms, preferably three or more, which may be straight, branched and/or contain a cyclic moiety, and may be alkyl (ethyl, or preferably propyl, butyl, including t-butyl, and so forth), aryl, or arylalkyl. These compounds include those CsA analogs which  
25 contain NMeLeu, NMelle, NMePhe or specifically the unnatural NMe[betaMePhe], in place of MeVal11. The "(b)" CsA compounds are of formula 2 where R represents a functional group as discussed above.



1 (R = Me) : CsA

2 (R ≠ Me) : Modified [MeVal<sup>11</sup>]CsA

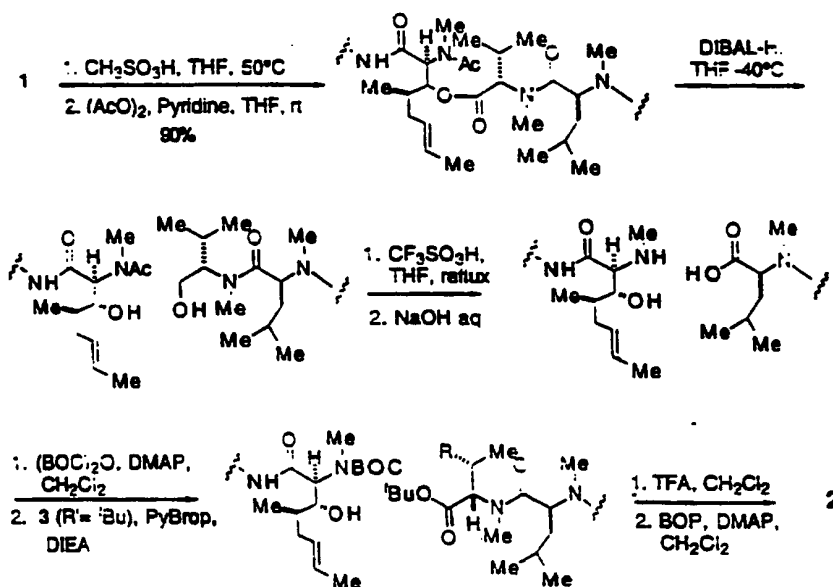


3

This invention further encompasses homo- and hetero-dimers and higher order oligomers containing one or more such CsA analogs. Preferably the compounds and oligomers comprising them in accordance with this invention bind to natural, or preferably mutant, cyclophilin proteins with an affinity at least 0.1% and preferably at least about 1% and even more preferably at least about 10% as great as the affinity of CsA for cyclophilin.

A two step strategy may be used to prepare the modified [MeVal<sup>11</sup>]CsA derivatives starting from CsA. In the first step the residue MeVal<sup>11</sup> is removed from the macrocycle. In the second step a selected amino acid is introduced at the (former) MeVal<sup>11</sup> site and the linear peptide is cyclized. The advantage of this strategy is the ready access to several modified [MeVal<sup>11</sup>]CsA derivatives in comparison with a total synthesis. The synthetic scheme is as follows:

102



- To differentiate the amide bonds, an *N,O* shift has been achieved between the amino and the hydroxyl groups from MeBmt1 to give IsoCsA (Ruegger et al., *Helv. Chim. Acta* 59 4 (1976): 1075-92) (see scheme above). The reaction was carried out in THF in the presence of methanesulfonic acid. (Oliyai et al., *Pharm Res* 9 5 (1992): 617-22). The free amine was protected with an acetyl group with pyridine and acetic anhydride in a one-pot procedure. The overall yield of the *N*-acetyl protected IsoCsA is 90%. The ester MeBmt1-MeVal11 bond is then reduced selectively in the presence of the *N*-methyl amide bonds, e.g. using DIBAL-H. The resulting diol is then transformed to the corresponding di-ester with another acid-induced *N,O* shift. This will prepare both the *N*-acetyl group and MeVal11 residues for removal through hydrolysis of the newly formed esters with aqueous base.

- After protection of the free amino group the new amino acid residue is introduced e.g. with the PyBrop coupling agent. Deprotection and cyclization of the linear peptide with BOP in presence of DMAP (Alberg and Schreiber, *Science* 262 5131 (1993): 248-250) completes the synthesis of 2. The binding of bumped-CsAs to cyclophilins can be evaluated by the same methods described for FK506s and FK1012s. Once cyclophilins are identified with compensatory mutations, bumped (CsA)<sub>2</sub> HED and HOD reagents may be synthesized



according to the methods discussed previously. Of particular interest are bumped CsA compounds which can form dimers which themselves can bind to a cyclophilin protein with 1:2 stoichiometry. Homo dimers and higher order homo-oligomers, heterodimers and hetero-higher order oligomers containing at least one such CsA or modified CsA moiety may be designed and evaluated by the methods developed for FK1012A and (CsA)<sub>2</sub>, and optimize the linker element in analogy to the FK1012 studies.

Mutant cyclophilins that bind our position 11 CsA variants (2) by accommodating the extra bulk on the ligand may be now be prepared. Cyclophilins with these compensatory mutations may be identified through the structure-based site-directed and random mutagenesis/screening protocols described in the FK1012 studies.

It is evident from the above results, that the subject method and compositions provide for great versatility in the production of cells for a wide variety of purposes. By employing the subject constructs, one can use cells for therapeutic or experimental purposes, where the cells may remain inactive until needed, and then be activated by administration of a safe drug. Because cells can have a wide variety of lifetimes in a host, there is the opportunity to treat both chronic and acute indications so as to provide short- or long-term protection. In addition, one can provide for cells which will be directed to a particular site, such as an anatomic site or a functional site, where therapeutic effect may be provided.

Cells can be provided which will produce a wide variety of proteins or other gene products which may serve to correct a deficit or inhibit an undesired result, such as activation of cytolytic cells, to inactivate a destructive agent, to kill a restricted cell population, or as is the focus here, to provide regulatable obstruction of the expression of a target gene or functioning of the target gene product. By having the cells present in a host over a defined period of time, the cells may be readily activated by administering the multimerizing drug at a dose which can result in a rapid response of the engineered cells. Cells can be provided where the expressed chimeric receptor is intracellular, avoiding immune response due to a foreign protein on the cell surface.

Furthermore, the intracellular chimeric receptor protein provides for efficient signal transduction upon ligand binding, apparently more efficiently than the receptor binding at an extracellular receptor domain.

5 By using relatively simple molecules which bind to chimeric membrane bound receptors, resulting in the expression of products of interest or inhibiting the expression of products, one can provide for models for the study of disease and for cellular therapeutic treatment. The multimerizing and related agents which may be administered are safe, can be administered in a variety of ways, and can ensure a very specific response, so as not to upset homeostasis.

10 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

15 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## Claims

1. Chimeric responder proteins comprising one or more ligand-binding domains and one or more action domains, which are capable of multimerizing in the presence of the ligand, and upon multimerization, are capable of activating transcription of a blocking gene under the transcriptional control of an expression control element which is activated in response to the presence of the ligand, wherein the blocking gene encodes an anti-sense message or ribozyme directed to a selected gene, a neutralizing antibody against a selected protein, a dominant negative form of a selected protein or the protein Cre.
2. A DNA construct encoding a chimeric responder protein of claim 1.
3. A cell containing and capable of expressing a DNA construct of claim 2.
4. A cell of claim 3 which further contains a DNA construct comprising a blocking gene under the transcriptional control of an expression control element which is activated in response to the presence of the ligand.
5. A cell of claim 4 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes a protein whose function leads to the elimination of a selected gene.
6. A cell of claim 5 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes the protein Cre.
7. A cell of claim 6 which further contains a target gene flanked by loxP sequence permitting elimination of the target gene in the presence of Cre.
8. A cell of claim 4 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes an anti-sense message.

9. A cell of claim 4 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes a ribozyme directed to a selected gene.
- 5 10. A cell of claim 4 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes a neutralizing antibody moiety directed against a selected protein.
11. A cell of claim 4 which further contains a DNA construct comprising a blocking gene, wherein the blocking gene encodes a dominant negative form of a selected protein.
- 10 12. An organism containing at least one cell of claim 4.
13. An organism of claim 12 wherein the organism is a mouse.
14. An organism of claim 12 which further contains a target gene flanked by loxP sequence permitting elimination of the target gene in the presence of Cre.
- 15 15. An organism of claim 14 wherein the organism is a mouse.

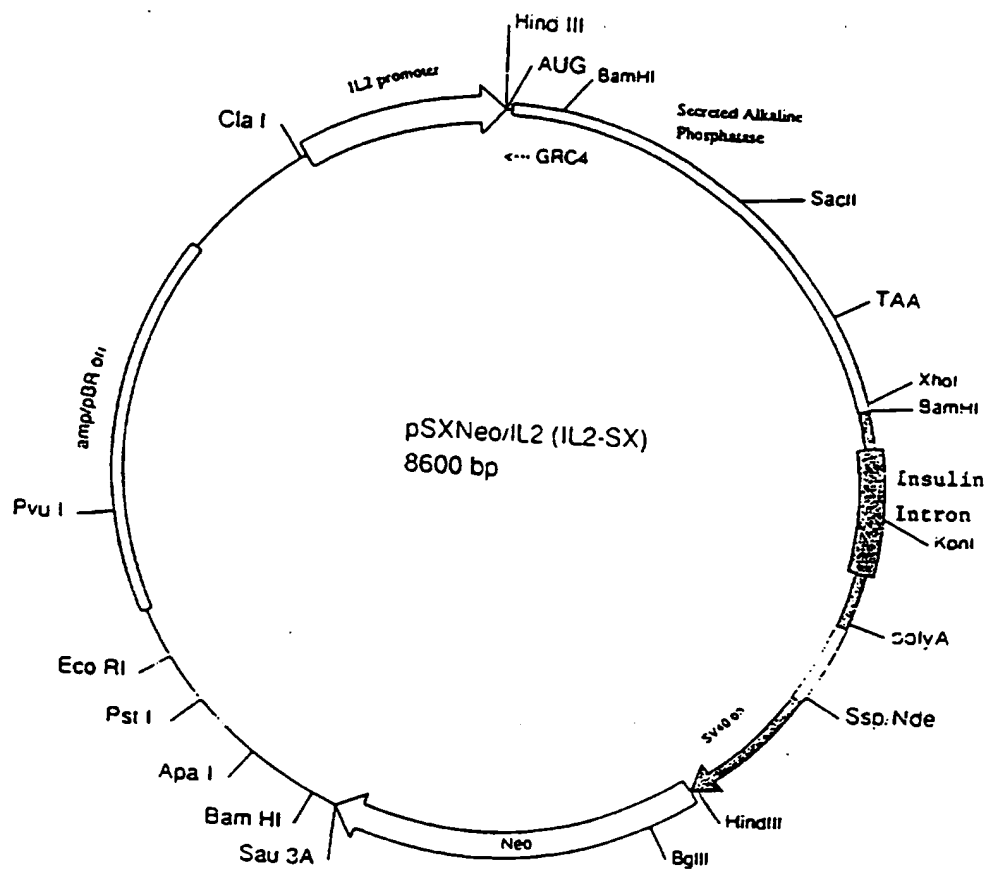
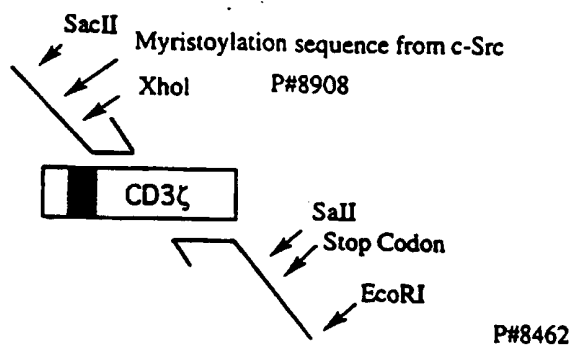


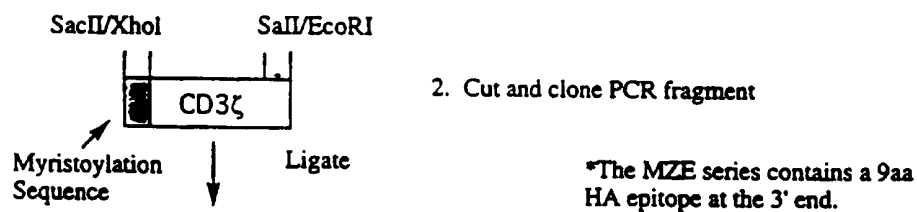
Figure 1/21

2/32

## Construction of intracellular signalling chimera:

1. PCR myristoylated CD3 $\zeta$ 

## 2. Cut and clone PCR fragment



## 3. SEQUENCE insert

## 4. Cut at XhoI or SalI and add FKBP domains

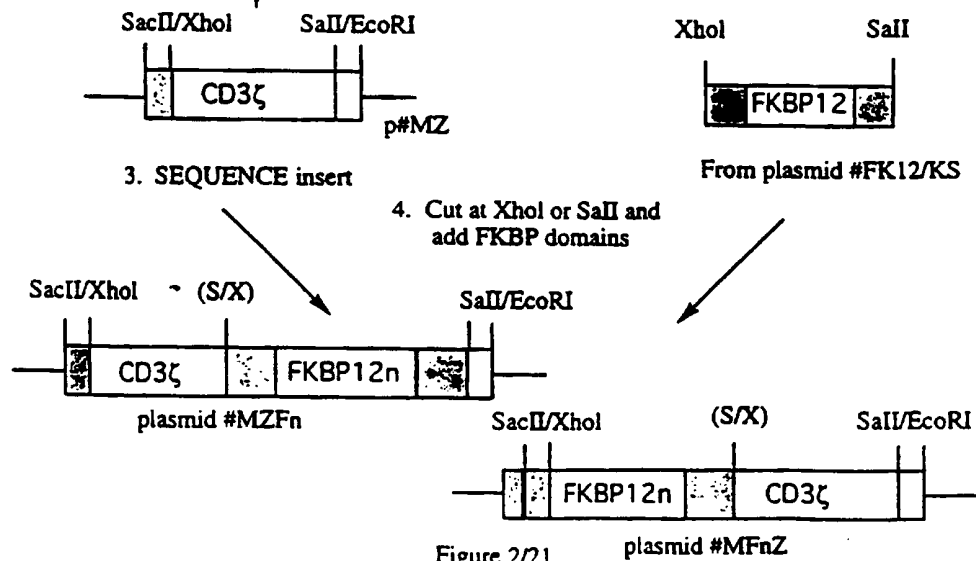
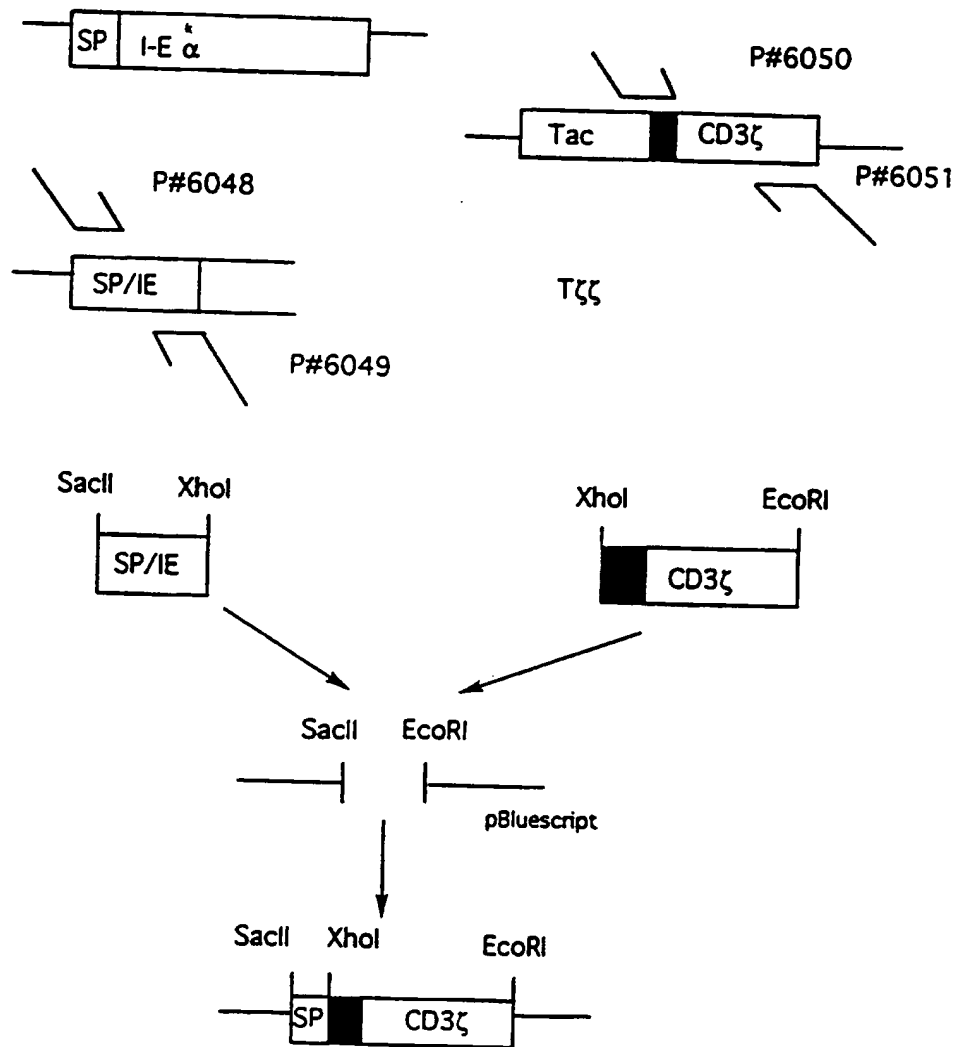


Figure 2/21

## Construction of extracellular signaling chimera:

1. PCR murine signal peptide

2. PCR CD3 trans-membrane and cytoplasmic domains

plasmid #SPZ/KS  
SEQUENCE insert\*

Cut XhoI

Figure 3A/21

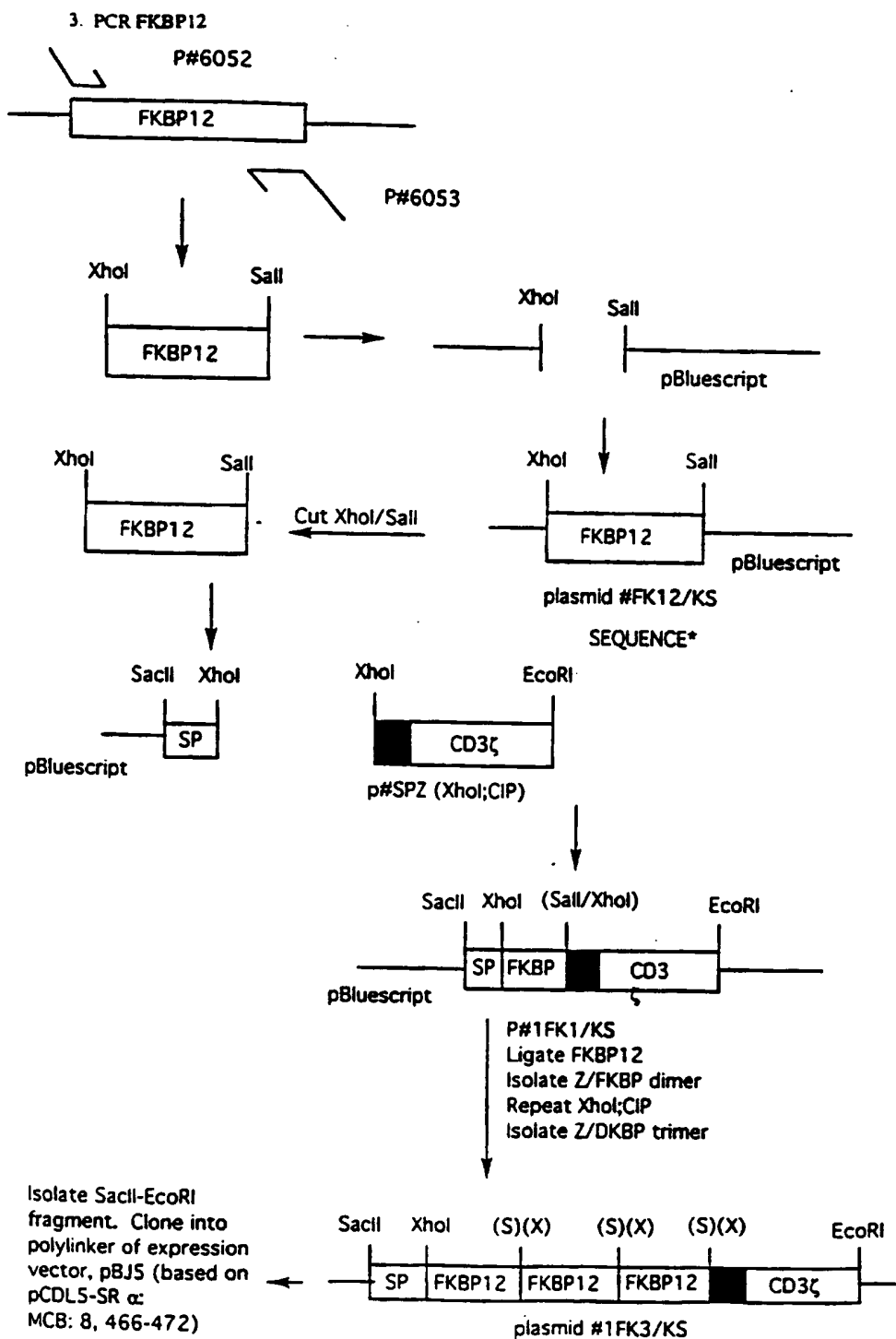


Figure 3B/21



5/32

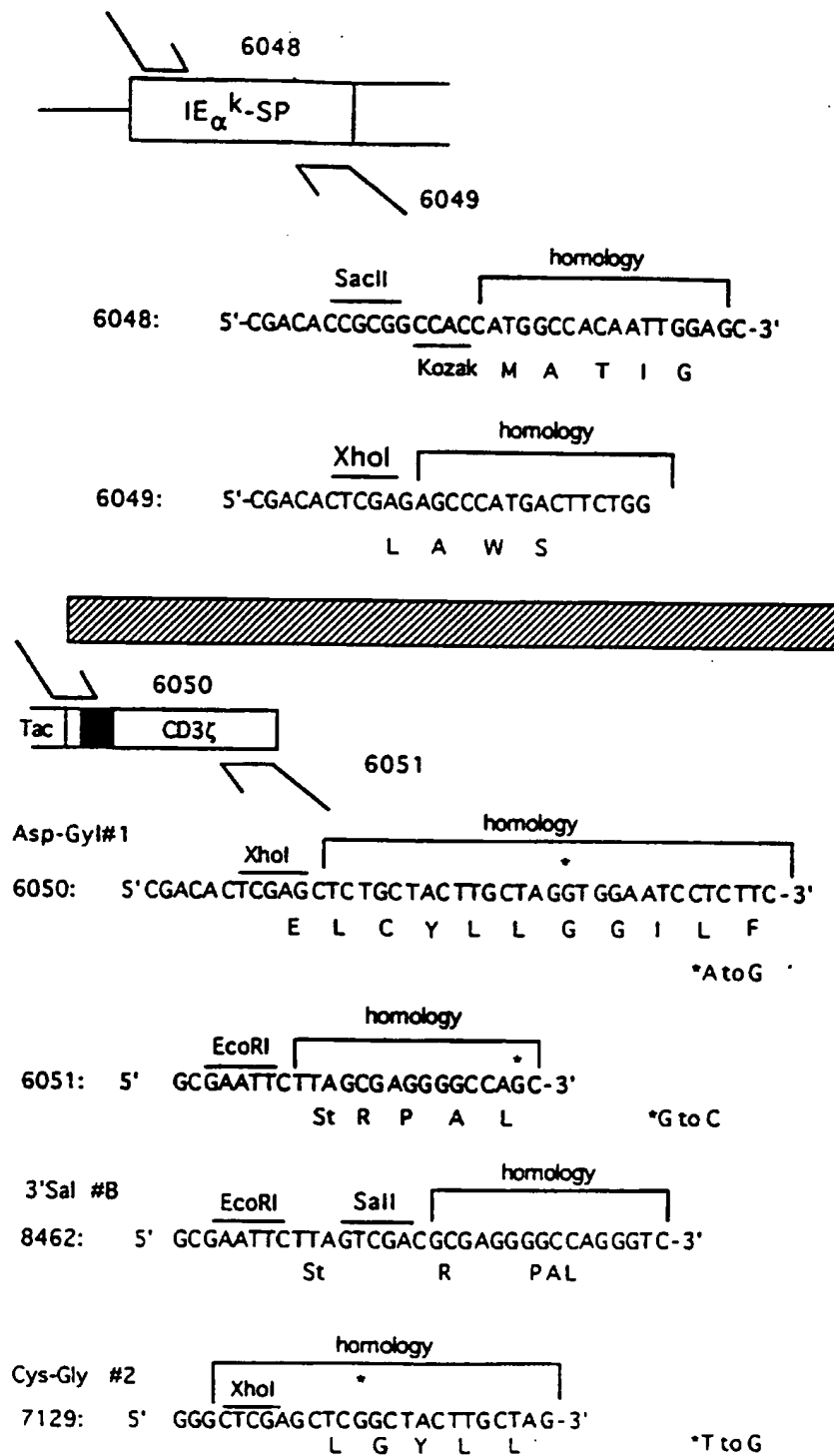


Figure 4A/21

CYCC

6568: 5'-CGACACTCGAGGTGACGGACAAGGTC-3'

XhoI      homology

6569: 5'-CGACAGTCGACCCAATCAGGGACCTC-3'

Sall      homology

EPITOPE

7850: 5'-TCGAGTATCGTACGACGTACCAGACTACGCAG-3'

XhoI      BsiWI

Y P Y D V P D Y A

7851: 5'-TCGACTGCGTAGTCTGGTACGTCGTACGGATAC-3'

Sall

EPITOPE: SSEP, 3XEP

8922: 5'-TCGACTATCCGTACGACGTACCAAGACTACGCAC-3'

Sall

8923: 5'-TCGAGTGCGTAGTCTGGTACGTCGTACGGATAG-3'

XhoI

Myristoylation from c-src SSMXZ

8908: 5'-CGACACCGCGGCCACCATGGGGAGTAGCAAGAGCAAGCCT

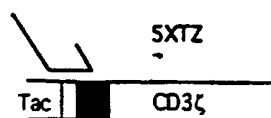
SacII

KOZAK M G S S K S K P

AAGGACCCAGCCAGCGCCTCGAGAGGAGTGCAGAGACTG-3'

XhoI      ζ-homology

K D P S Q R L E R S A E T



8912: 5'-CGACACTCGAGGAGCTCTGTGACGATG-3'

XhoI      homology

E L C D D

Figure 4B/21

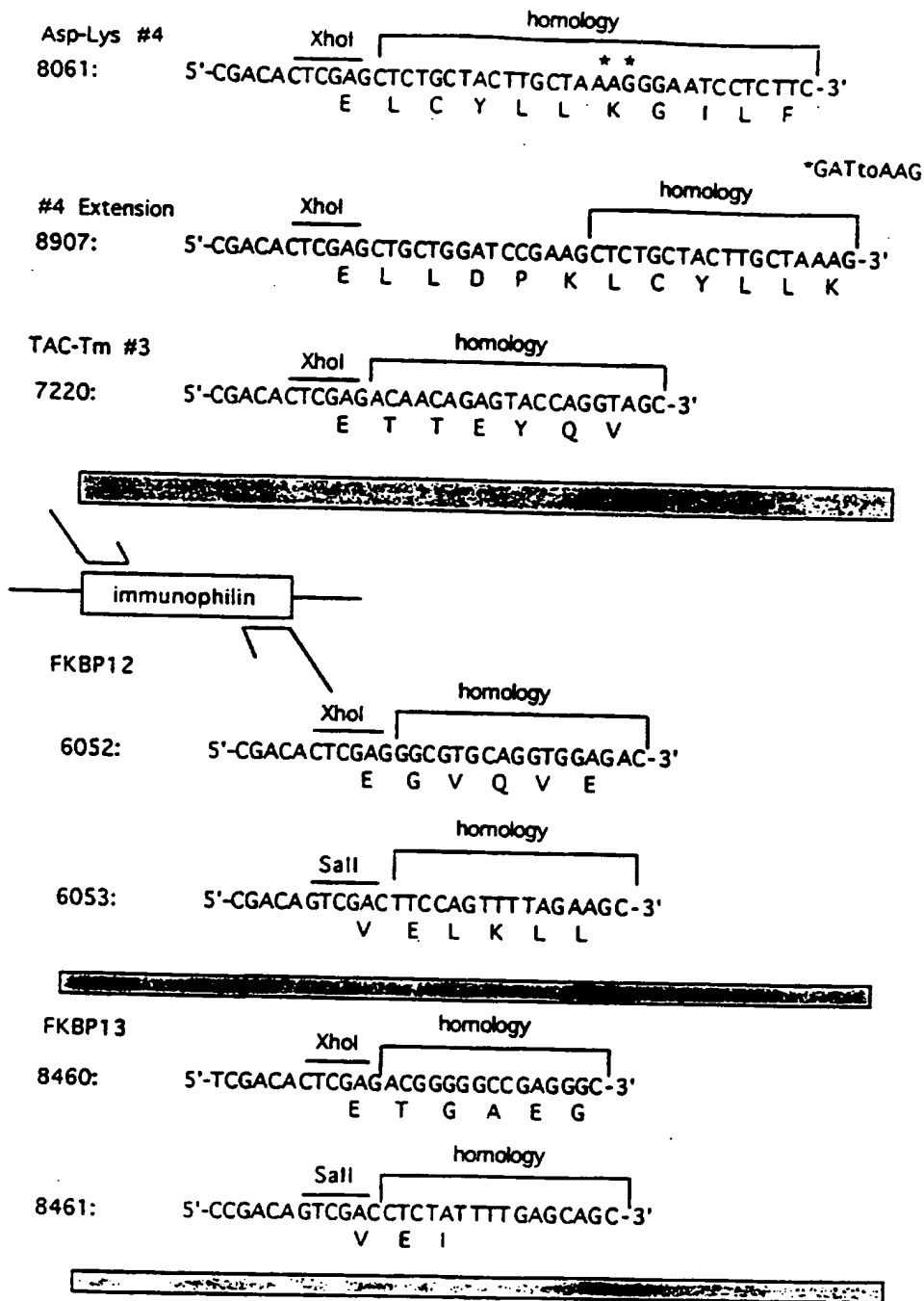


Figure 4C/21

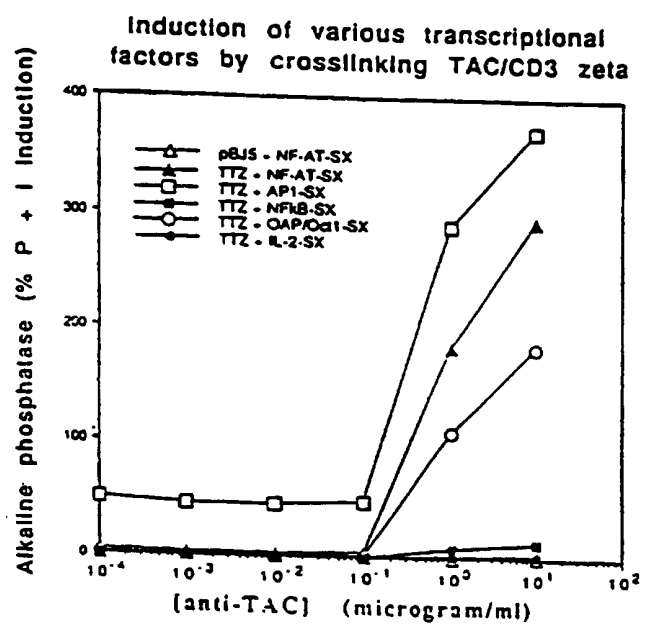


Figure 5/21

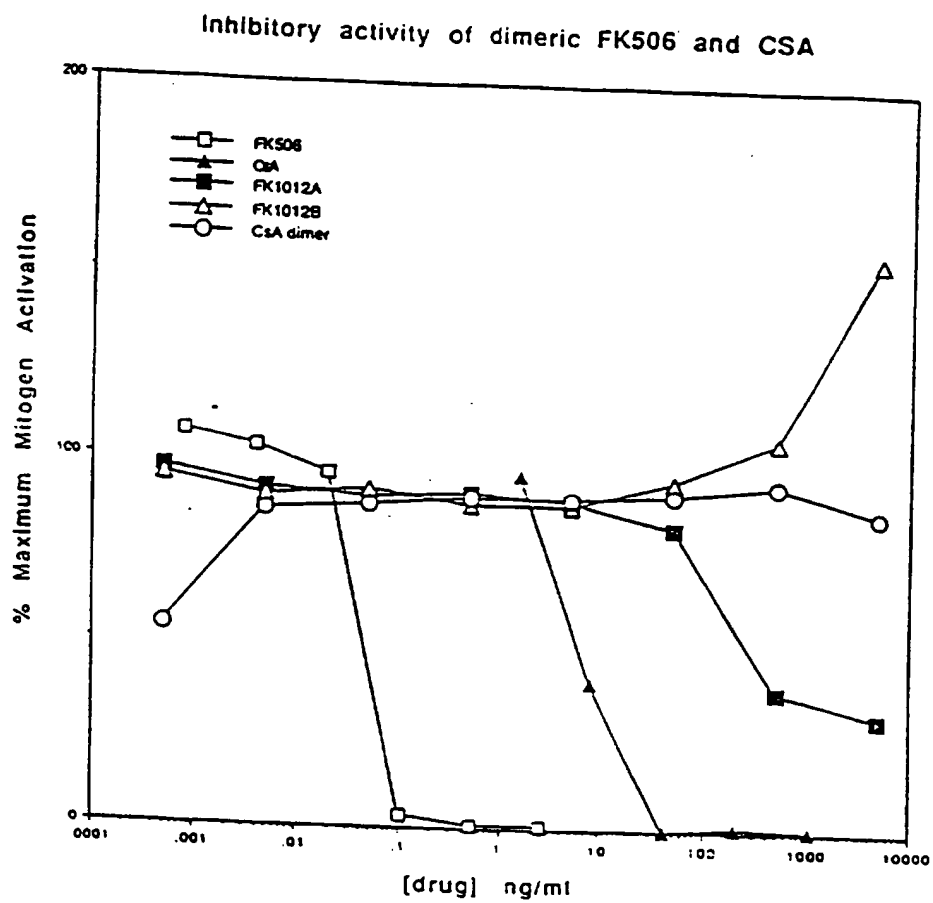
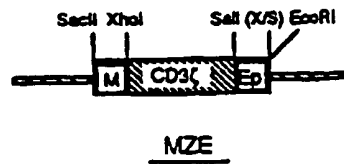
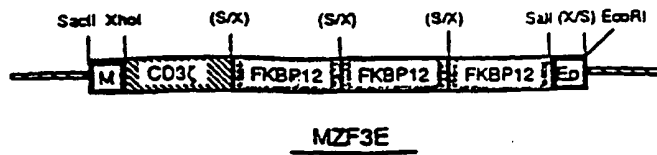
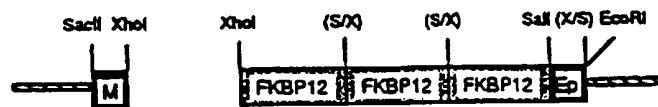
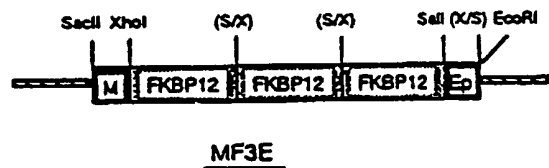


Figure 6A/21

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Cut Xho/SalI; CIP; + FKBP12 X 3



+



1. Cytoplasmic moiety of surface receptor
2. Tyrosine Kinase
3. Transcription Factor
4. Others

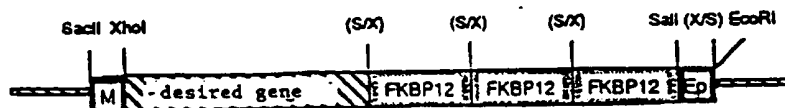


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Activity of FK1012A on the chimeric FKBPX3/CD3 zeta receptor

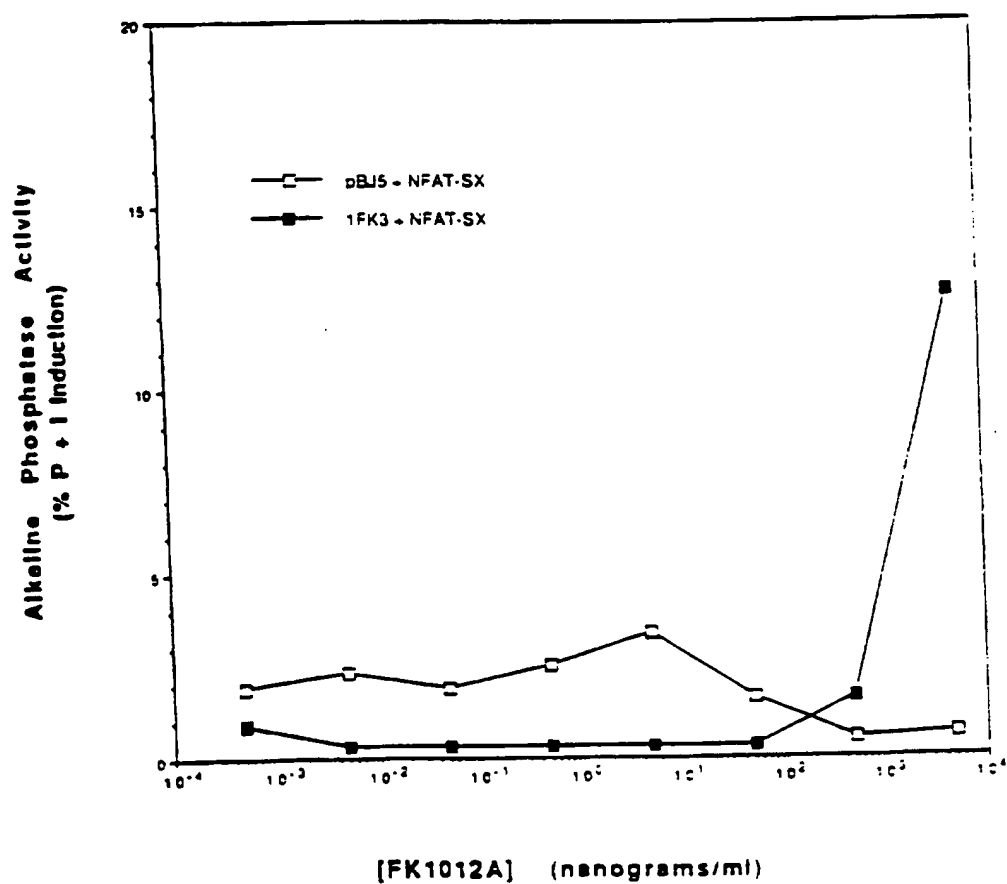


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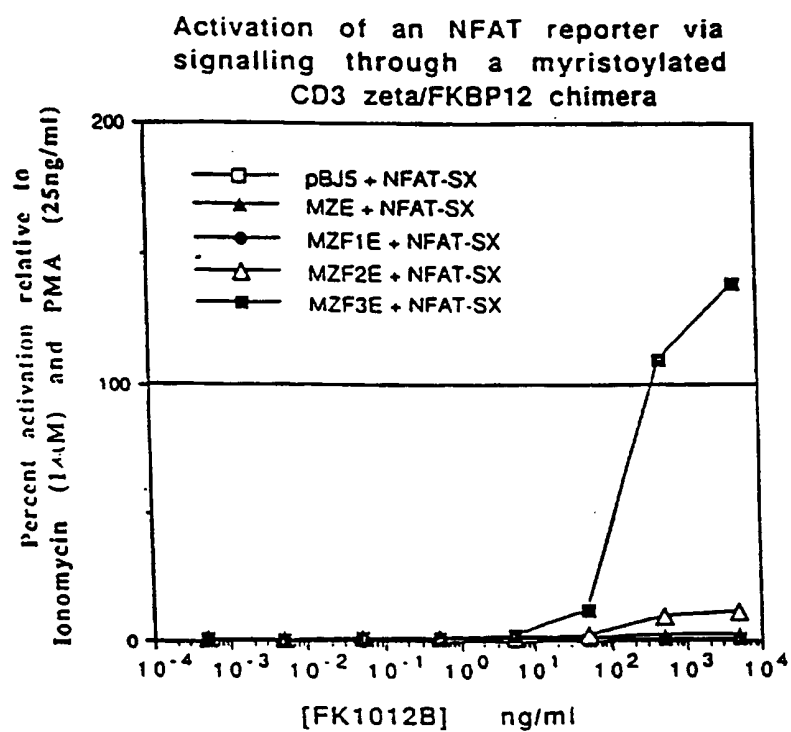


Figure 8/21



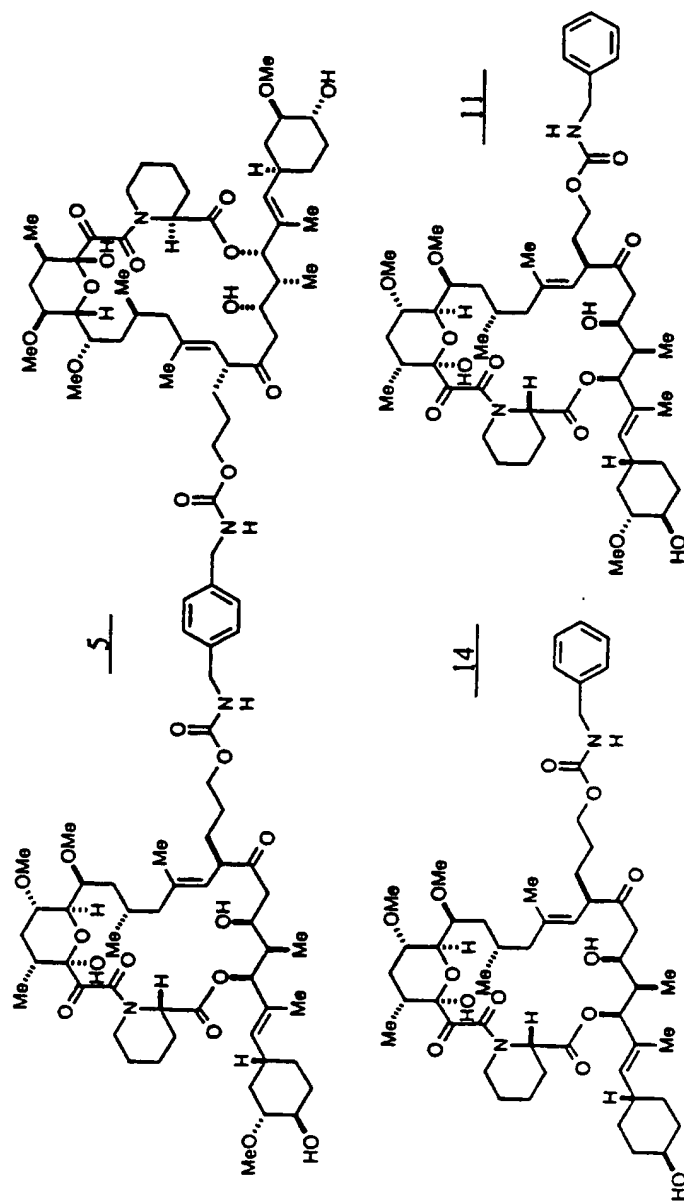


Figure 9A (#1)/21

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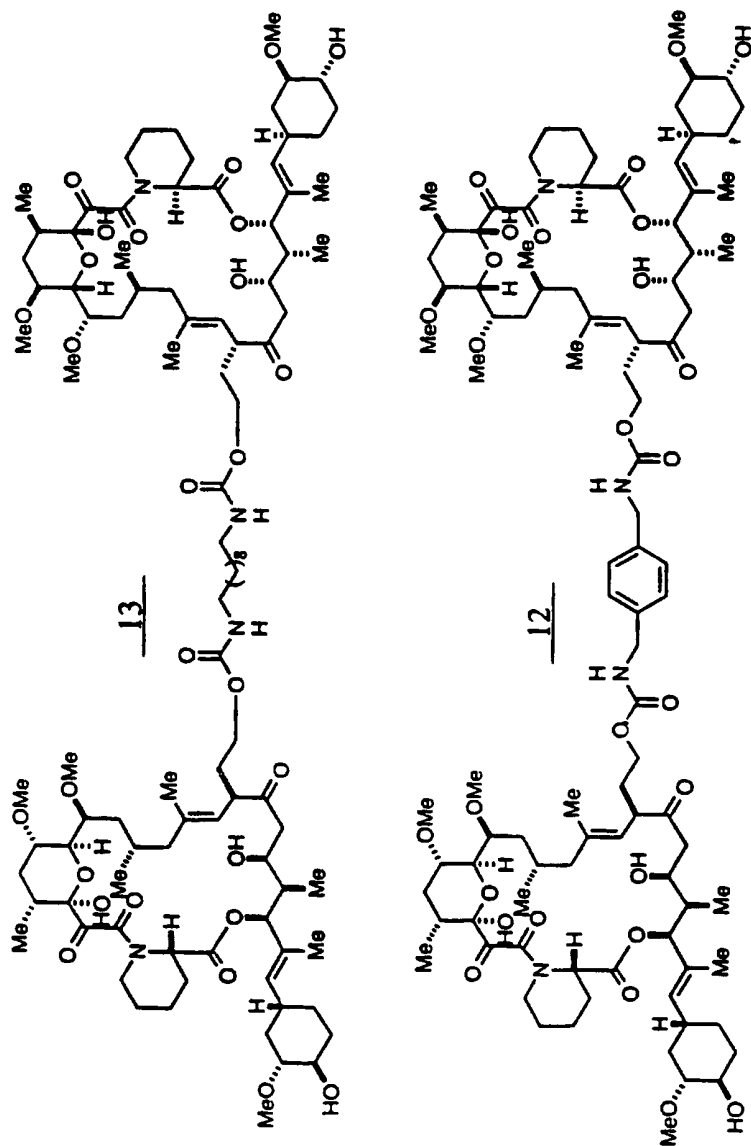


Figure 9A(#2)/21

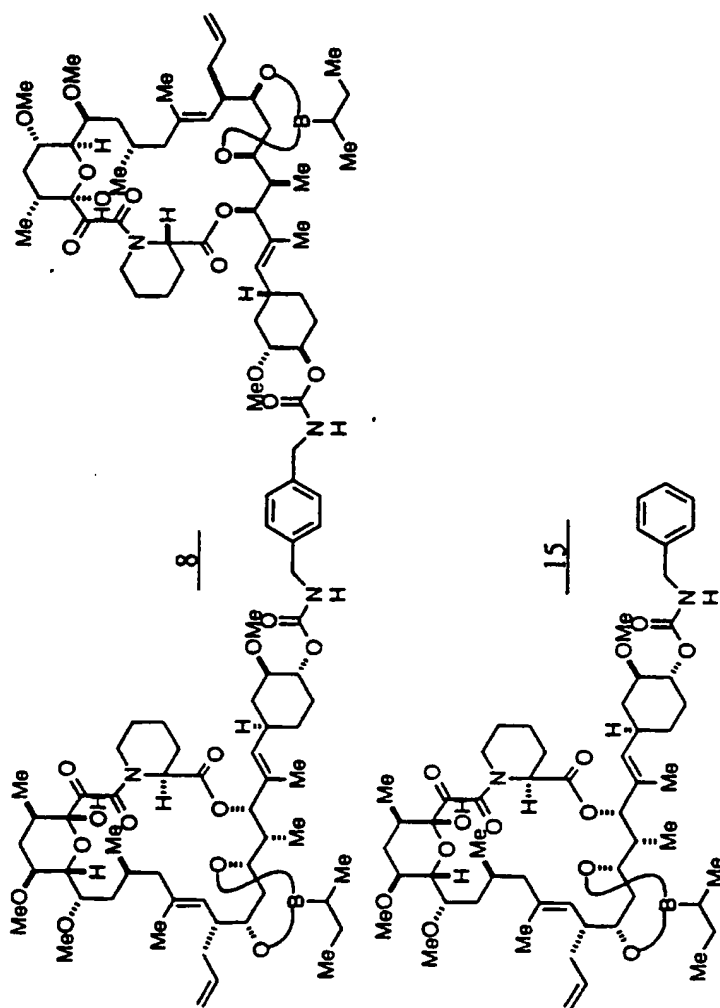


Figure 9B (#1)/21

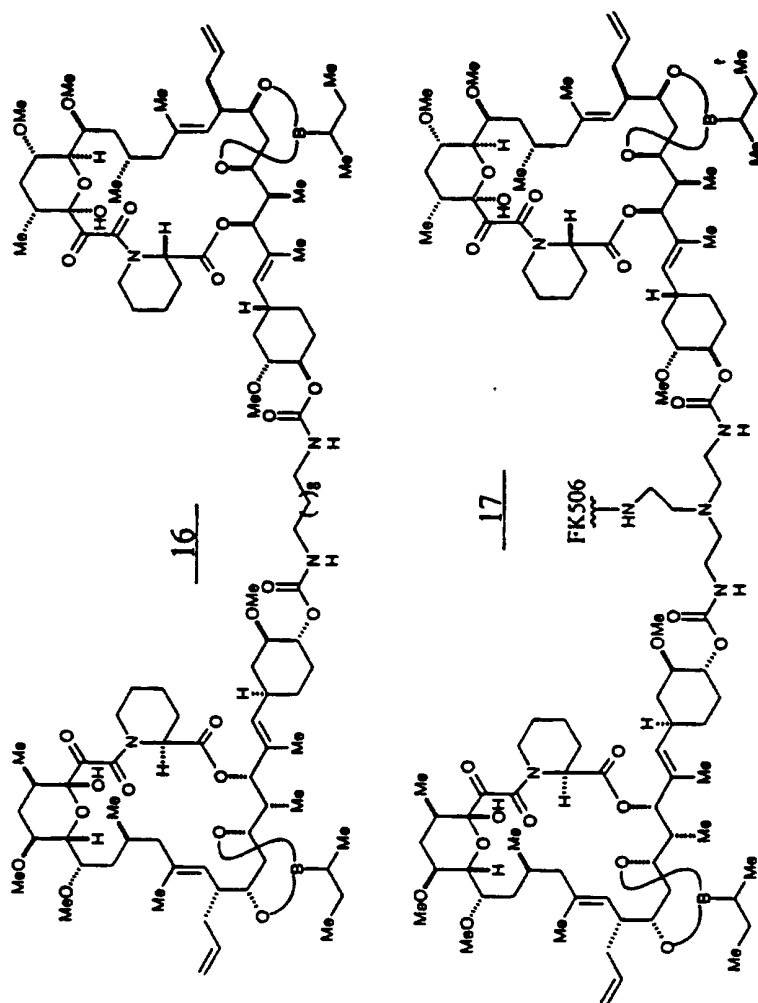


Figure 9B (#2)/21

## Scheme 1

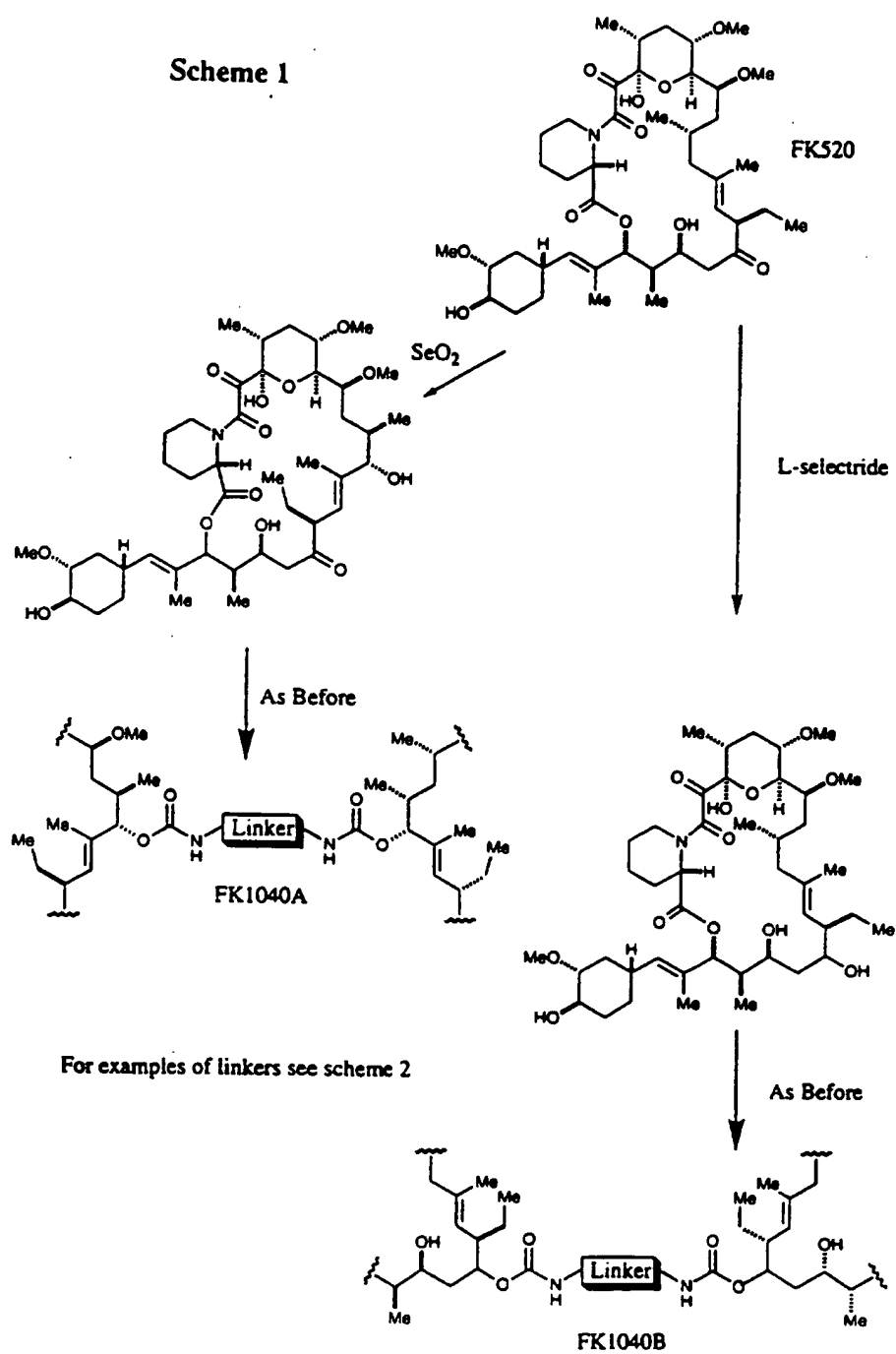


Figure 10/21

## Scheme 2: Synthesis of Dimers

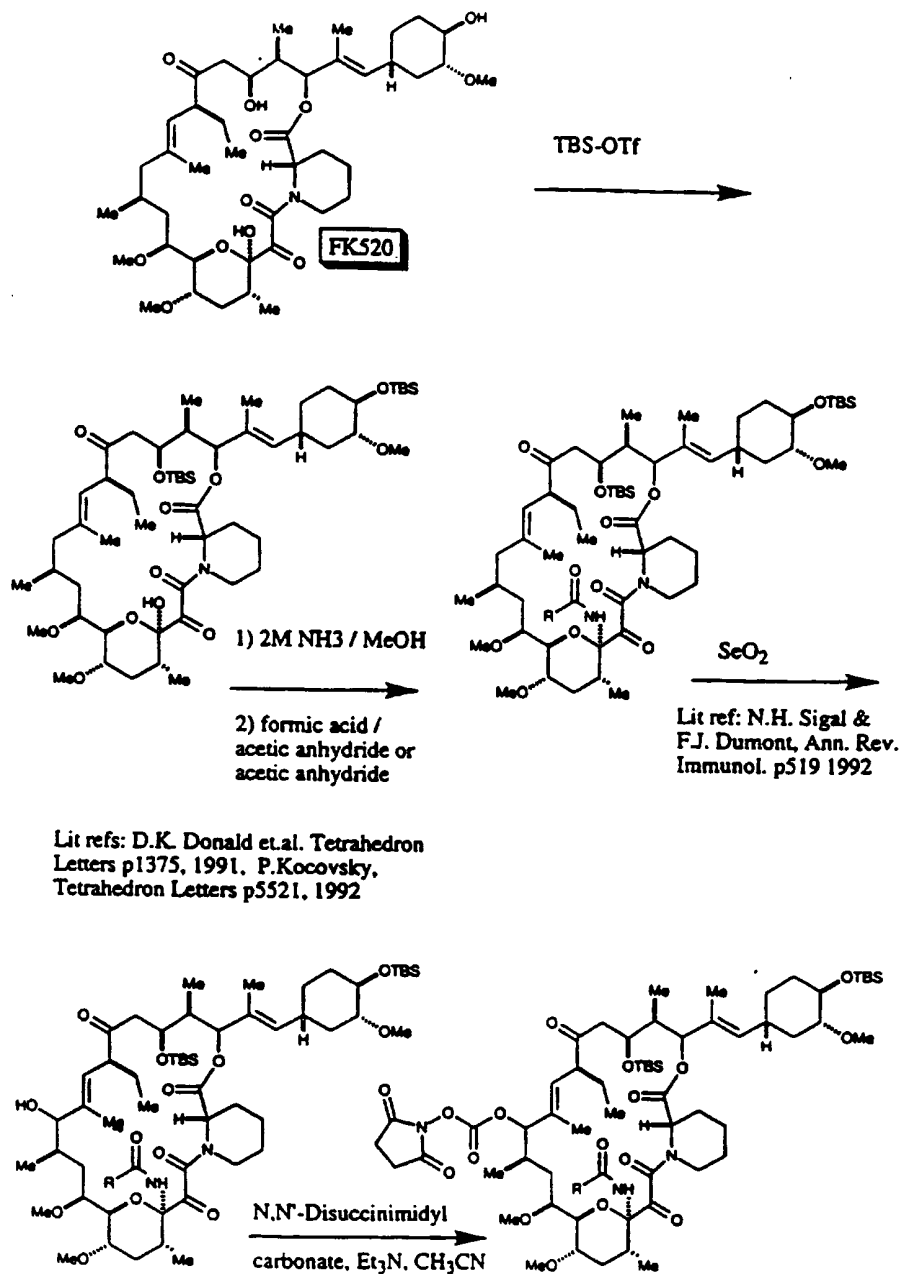


Figure 11A/21

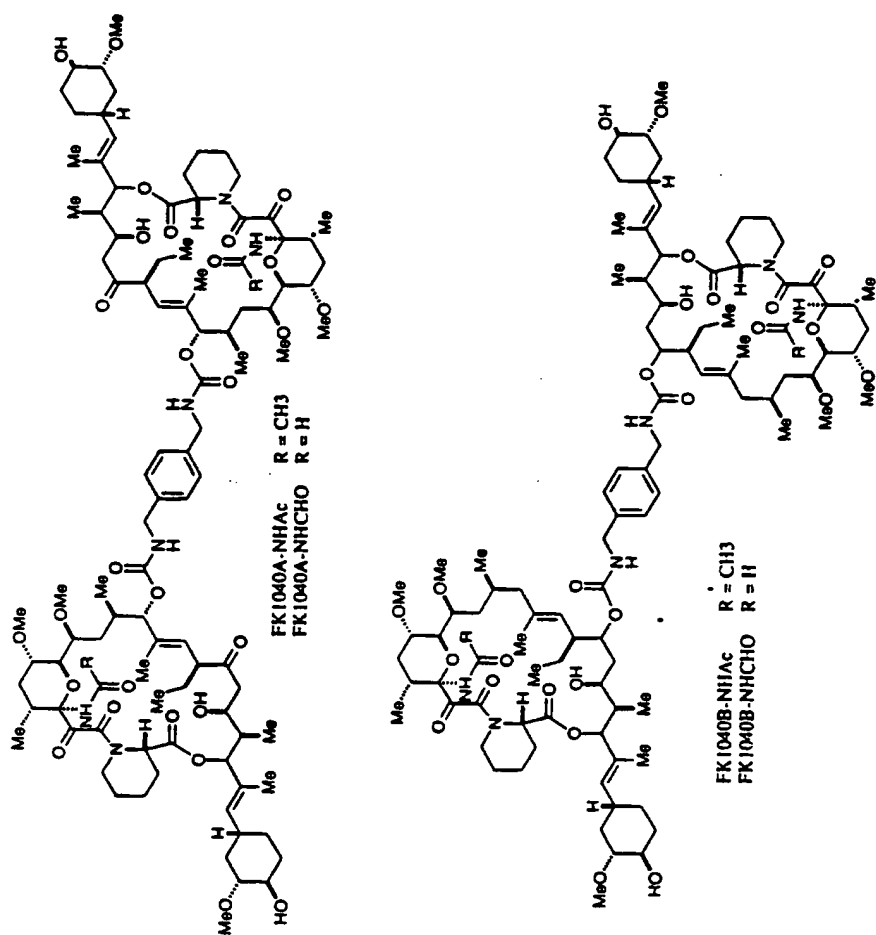


Figure 11B (#1)/21

An additional modified FK520 (FK1040) that interferes with FKBP12 yet should bind the FKBP12 mutant: F36A or F99A or Y26A, or combinations thereof is

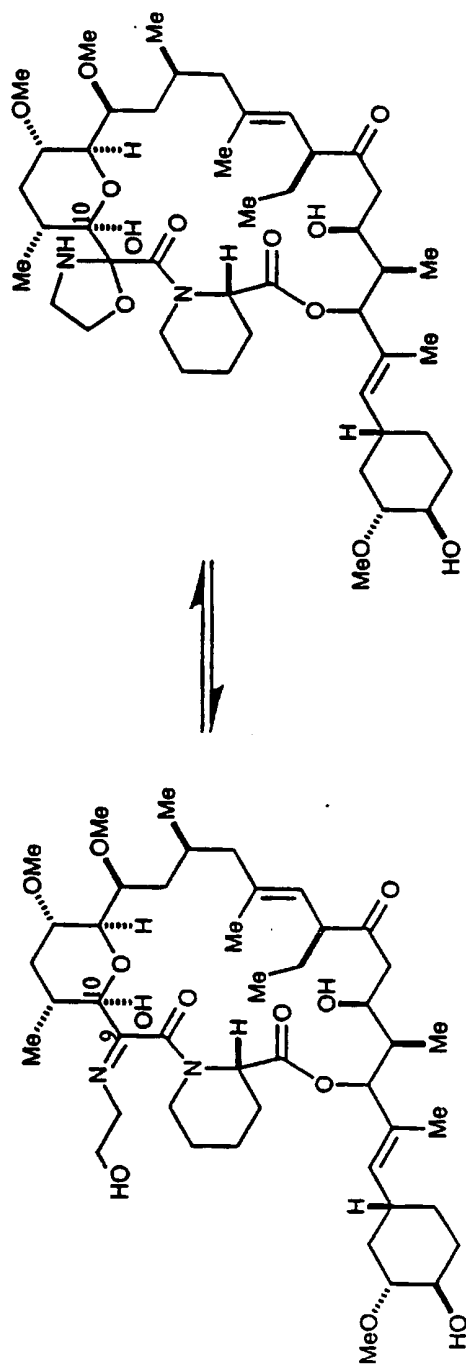


Figure 11B (#2)/21



## Scheme 3 Heterodimerization

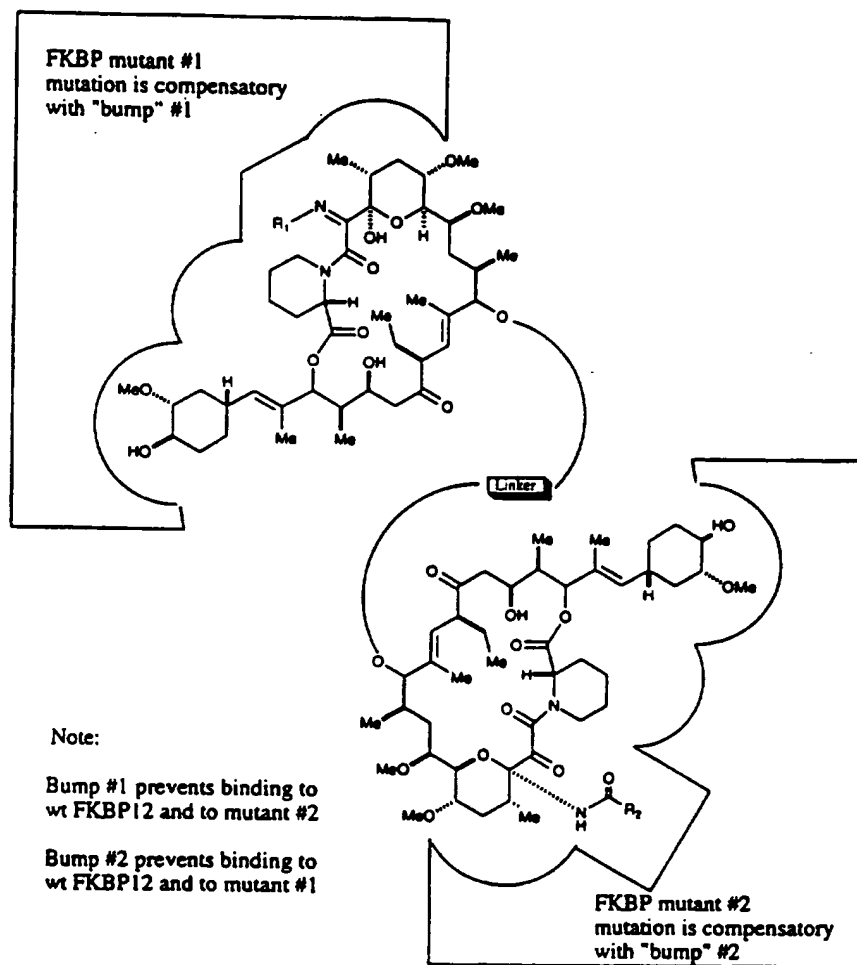
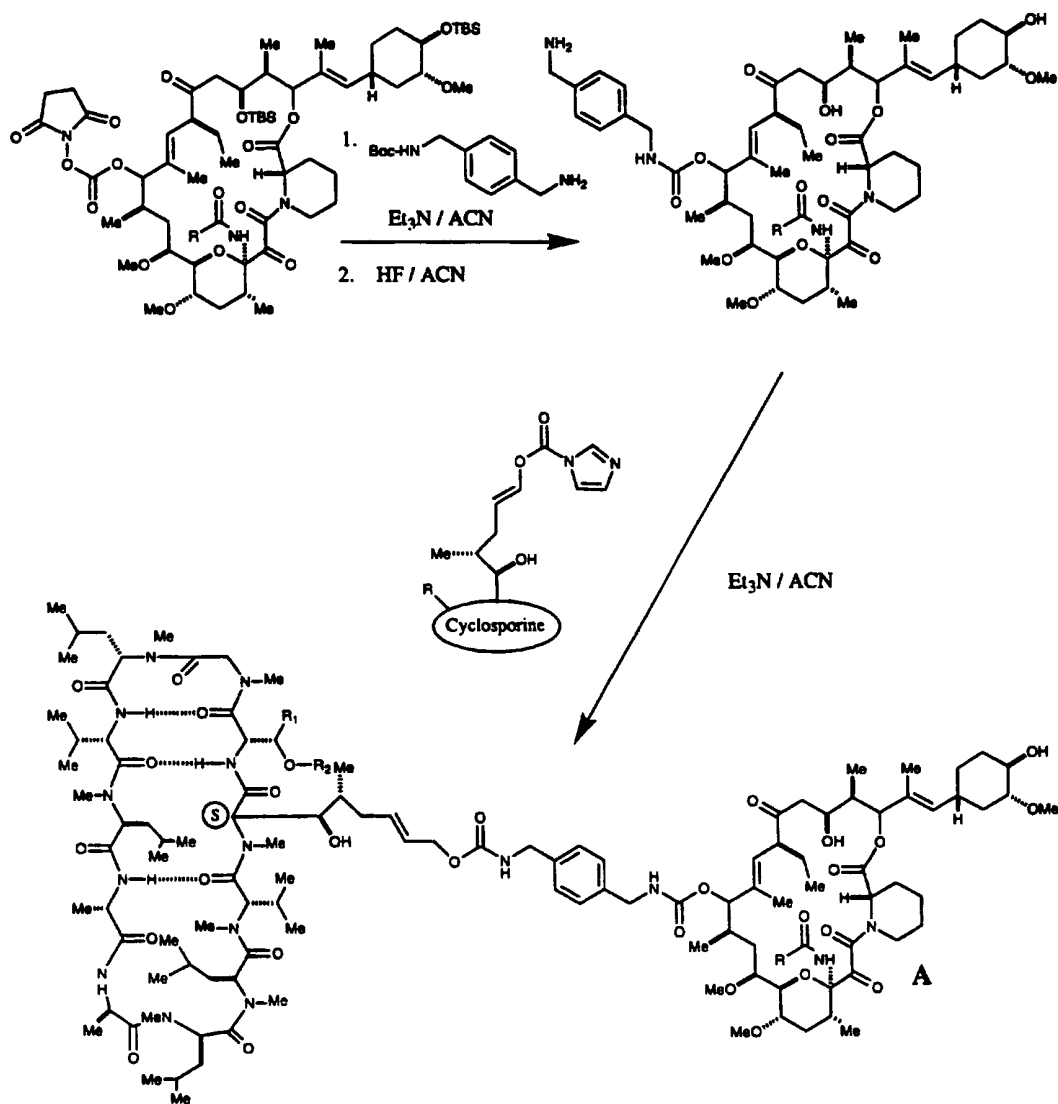


Figure 12/21

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## Scheme 3: Synthesis of heterodimers



In this example, a heterodimer of a cyclosporine analog and FK520A-NHCO-R were heterodimerized. However, the scheme can easily incorporate other FK506/520 derivatives to form hetero or homodimers

Figure 13/21

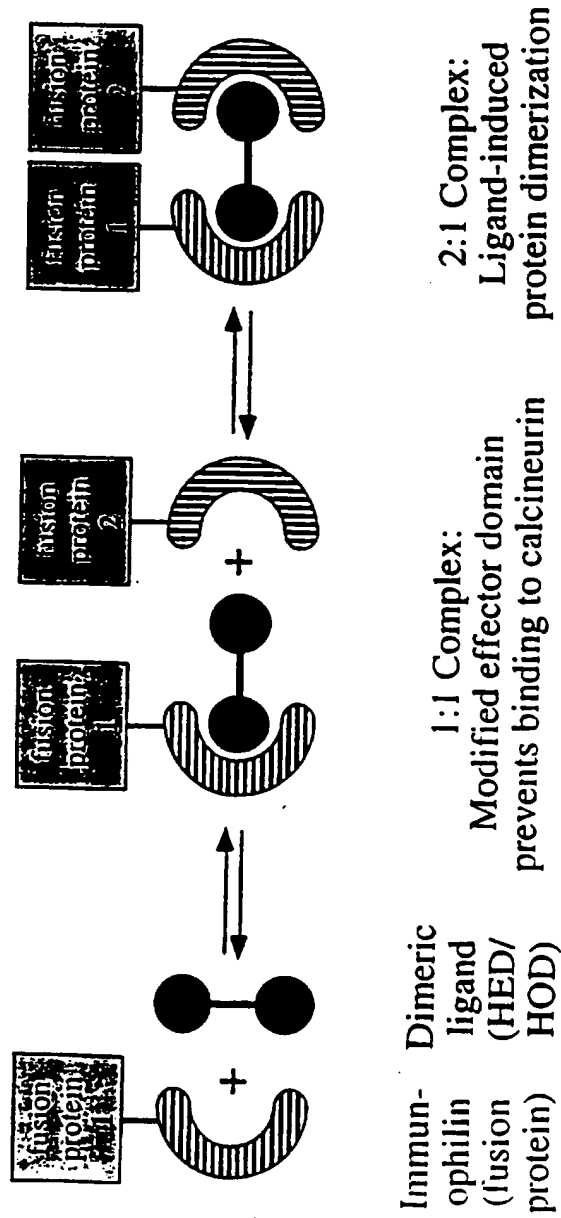


Figure 14/21

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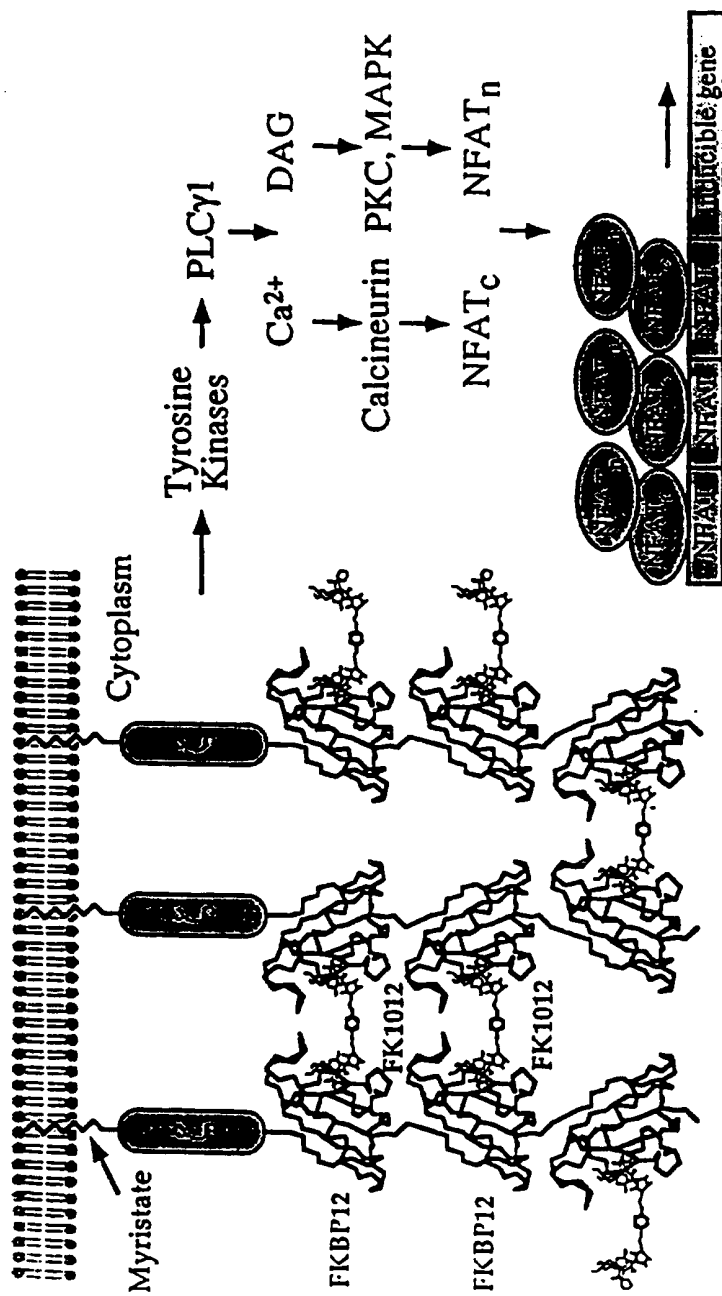
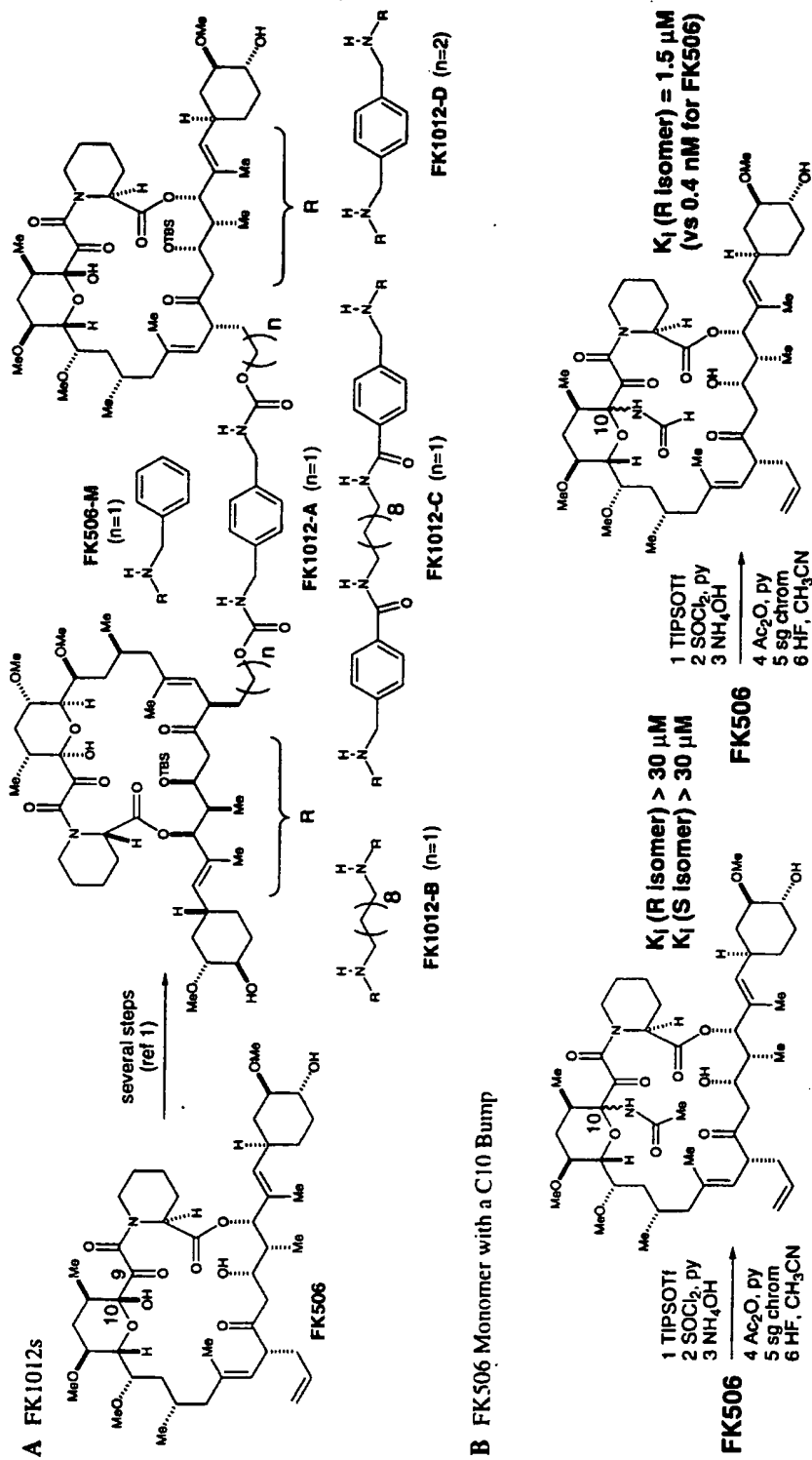


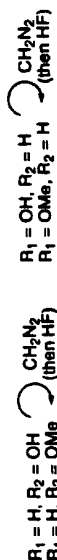
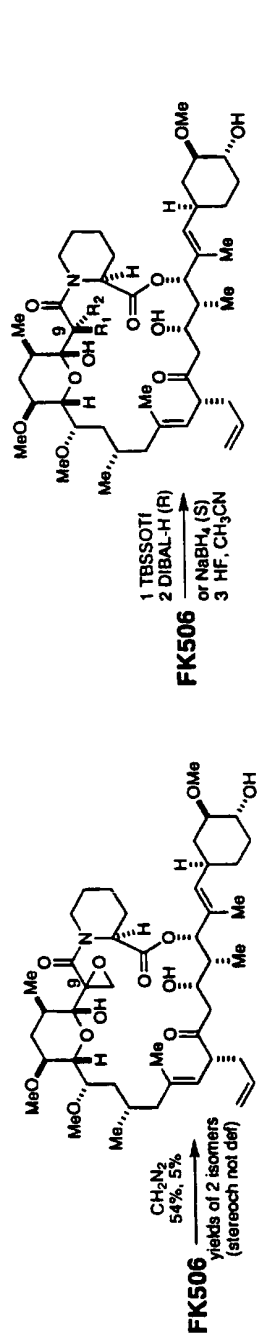
Figure 15/21



**Figure 16 (#1)/21**

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## C FK506 Monomer with a C9 Bump



## D HED Reagent Synthesis

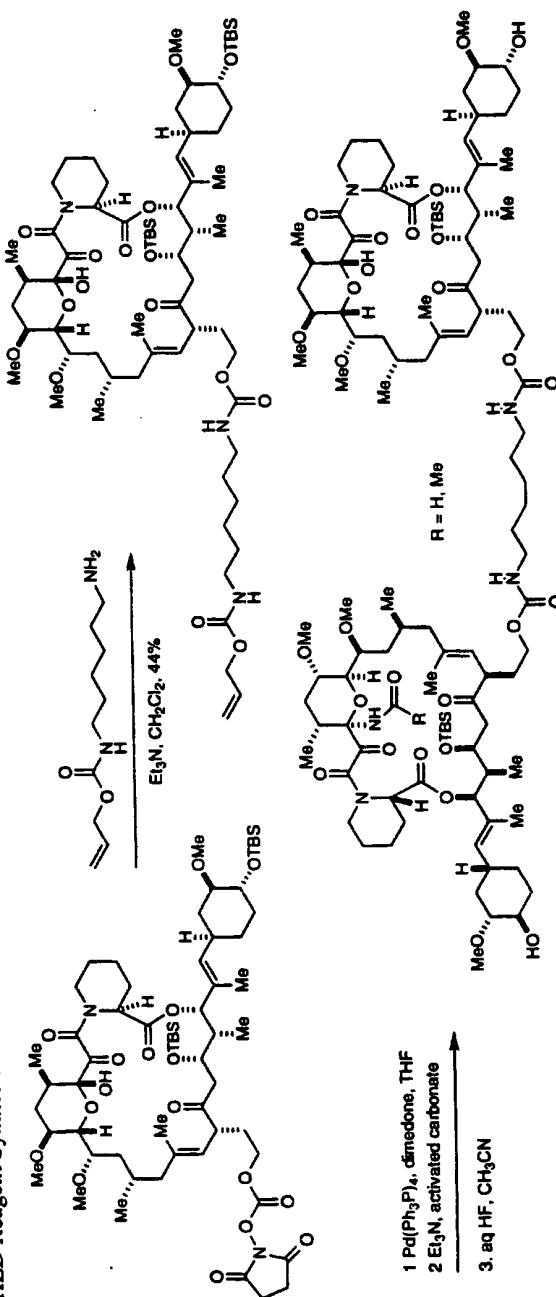


Figure 16 (#2)/21

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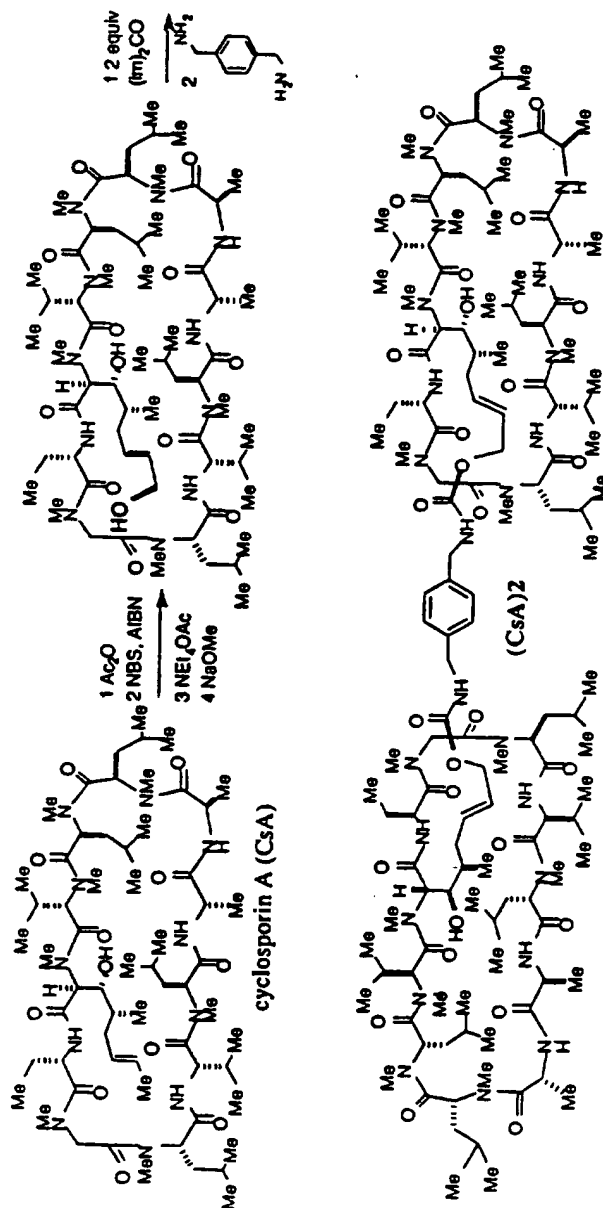


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**A** cDNA construct

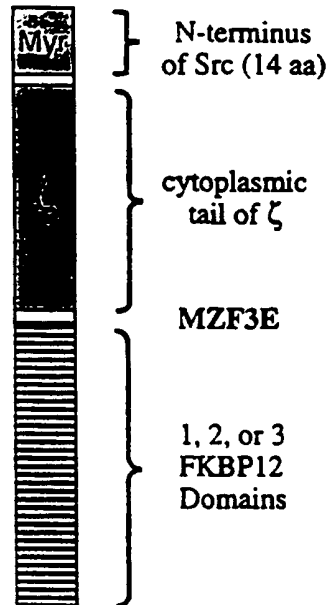


Figure 18A/21

**B** expressed protein

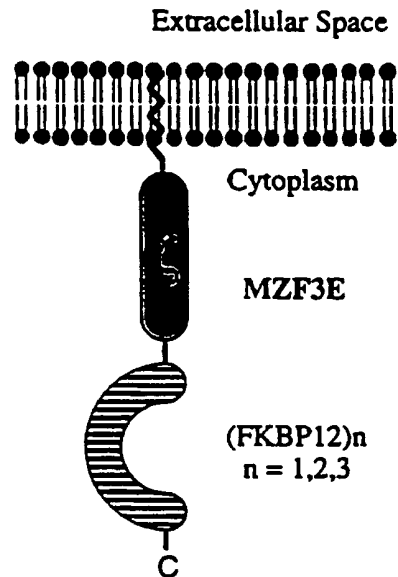


Figure 18B/21



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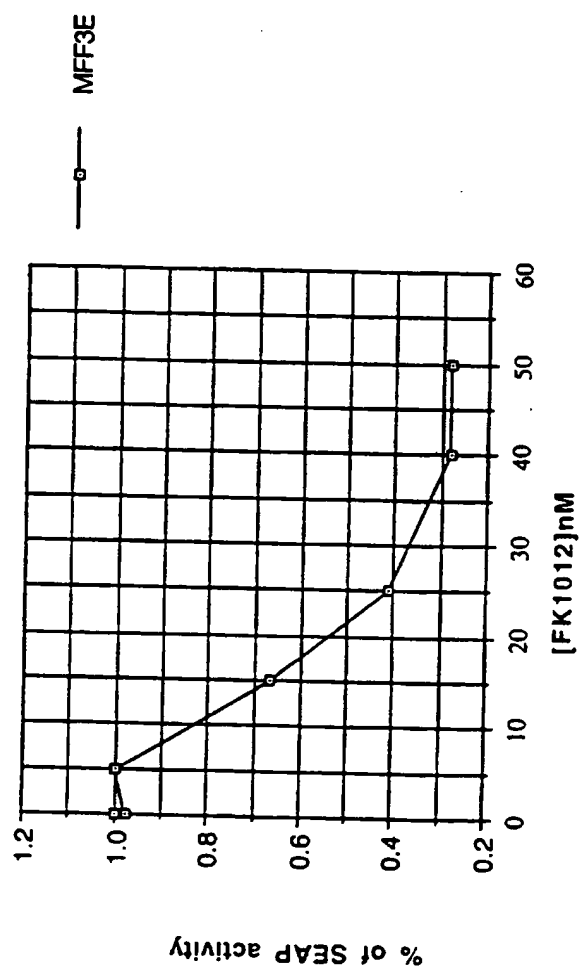


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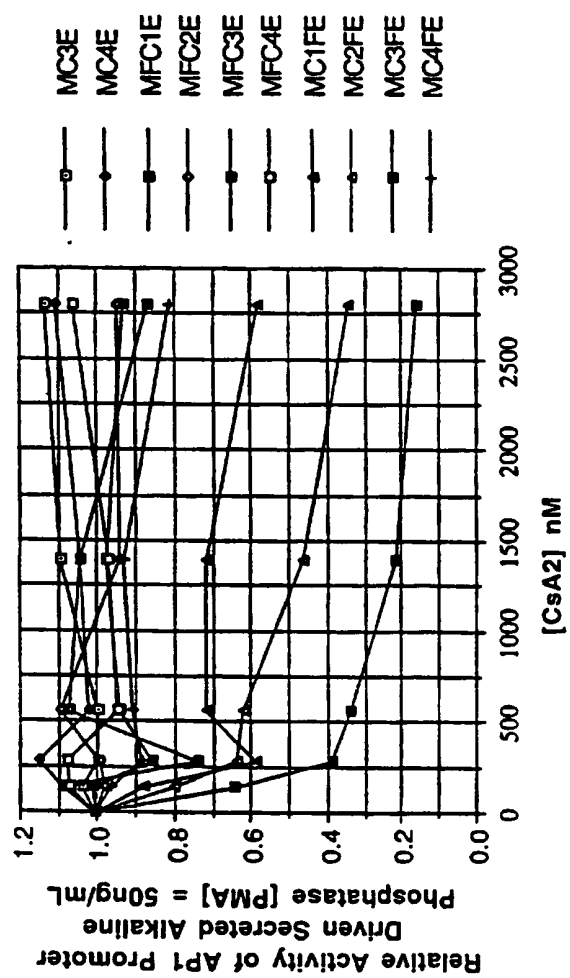


Figure 20A/21

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		LD50 Jurkat Cells	Relative Protein Expression
<b>A</b>			
MFF3E	Myr   Fas   FKBP   FKBP   Ep	15 nM	+
<b>B</b>			
MFC1E	Myr   Fas   CypC   Ep	NA	-
MFC2E	Myr   Fas   CypC   CypC   Ep	NA	-
MFC3E	Myr   Fas   CypC   CypC   Ep	NA	-
MFC4E	Myr   Fas   CypC   CypC   Ep	NA	-
MC1FE	Myr   CypC   Fas   Ep	500 nM	+
MC2FE	Myr   CypC   CypC   Fas   Ep	300 nM	+
MC3FE	Myr   CypC   CypC   Fas   Ep	200 nM	+
MC4FE	Myr   CypC   CypC   CypC   Fas   Ep	NA	+/-
MC3E	Myr   CypC   CypC   CypC   Ep	>30 uM	† +++
MC4E	Myr   CypC   CypC   CypC   Ep	>30 uM	++++

Figure 20B/21

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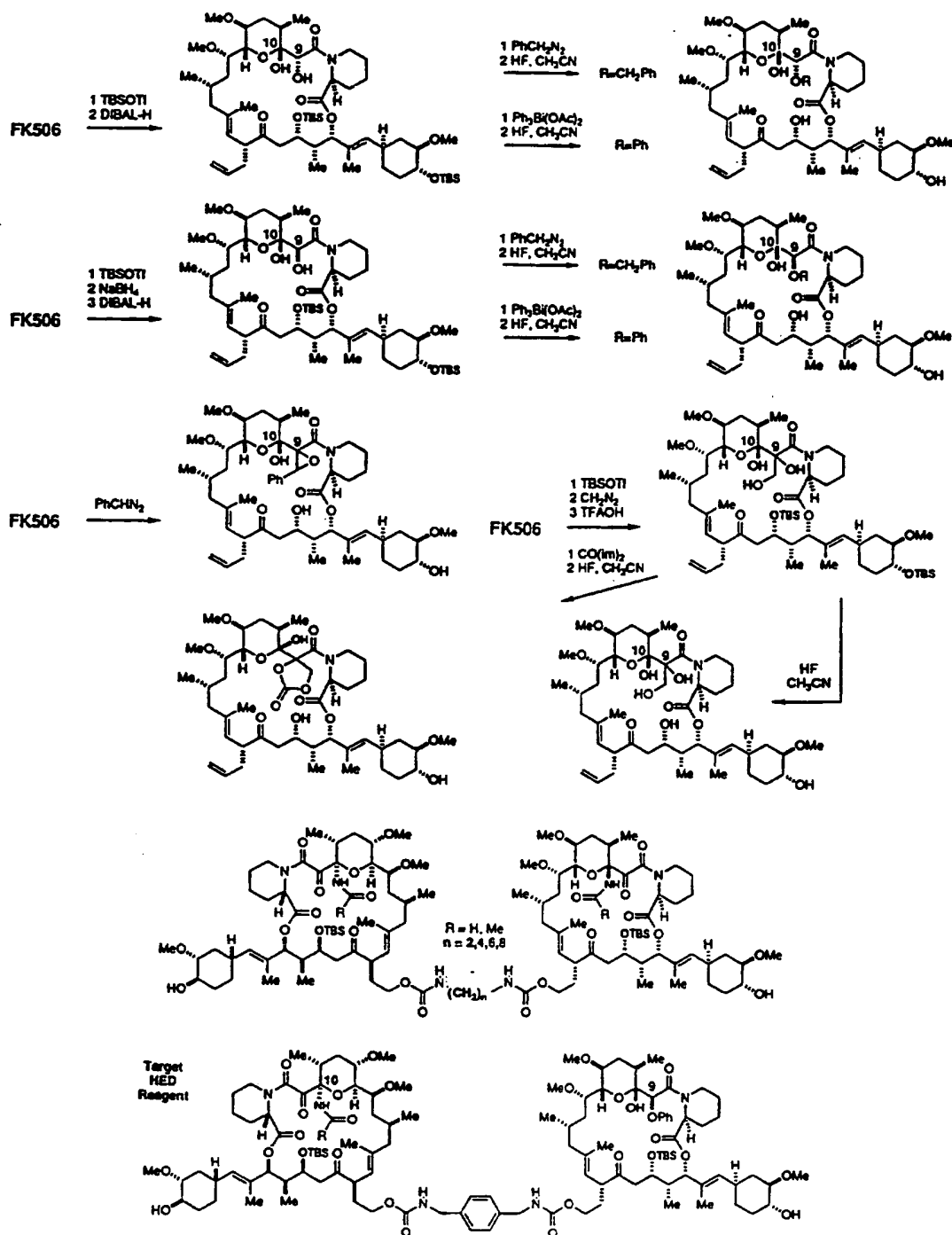


Figure 21/21

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/10591

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/00; C07H 21/00; C12N 5/00, 15/00

US CL : 530/350; 536/23.1; 435/172.3, 240.2, 320.1; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 435/172.3, 240.2, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: cre, lox P, transgenic, chimeric, ligand, recombinant, eliminate, inventor's name

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,959,317 (SAUER) 25 September 1990, see the entire document.	1-15
Y, P	Molecular and Cellular Biology, Volume 15, No. 3, issued March 1995, Qi et al., "The Ligand-Binding Domains of the Thyroid Hormone/Retinoid Receptor Gene Subfamily Function In Vivo To Mediate Heterodimerization, Gene Silencing, and Transactivation", pages 1817-1825, see the entire document.	1-15
Y	J. Mol. Biol., Volume 209, issued 1989, Carey et al., "An Amino-terminal Fragment of GAL4 Binds DNA as a Dimer", pages 423-432, see the entire document.	1-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

07 NOVEMBER 1995

Date of mailing of the international search report

06 DEC 1995

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/10591

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 54, issued 15 July 1988, Webster et al., "The Hormone-Binding Domains of the Estrogen and Glucocorticoid Receptors Contain an Inducible Transcription Activation Function", pages 199-207, see the entire document.	1-15
Y	Science, Volume 262, issued 12 November 1993, Spencer et al., "Controlling Signal Transduction with Synthetic Ligands", pages 1019-1024, see the entire document.	1-15
Y	Science, Volume 255, issued 03 January 1992, Letourneur et al., "Activation of T Cells by a Tyrosine Kinase Activation Domain in the Cytoplasmic Tail of CD3 ", pages 79-82, see the entire document.	1-15
Y	Science, Volume 265, issued 01 July 1994, Gu et al., "Deletion of a DNA Polymerase $\beta$ Gene Segment in T Cells Using Cell Type-Specific Gene Targeting", pages 103-106, see the entire document.	1-15
Y	Proc. Natl. Acad. Sci., Volume 89, issued August 1992, Orban et al., "Tissue- and site-specific DNA recombination in transgenic mice", pages 6861-6865, see the entire document.	1-15
Y	Proc. Natl. Acad. Sci., Volume 89, issued July 1992, Lakso et al., "Targeted oncogene activation by site-specific recombination in transgenic mice", pages 6232-6236, see the entire document.	1-15